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(57) Abstract

This invention provides an isolated nucleic acid molecule which encodes a wild–type or mutated SEL–10. This invention also provides a purified wild–type SEL–10 protein or a purified mutated SEL–10 protein. This invention also provides a method for production of an antibody capable of binding to wild–type SEL–10 or mutated SEL–10 protein. This invention also provides an antibody capable of specifically binding to wild–type SEL–10 or mutated SEL–10. This invention also provides a transgenic animal comprising the isolated nucleic molecule encoding SEL–10. This invention also provides a method for identifying a compound which is capable of ameliorating Alzheimer's disease. This invention also provides a method for determining whether a compound is capable of ameliorating Alzheimer's disease. This invention further provides a method for identifying a compound which is capable of treating cancer. This invention also provides a method for determining whether a compound is capable of treating cancer. This invention also provides a method for identifying a suppressor or enhancer that affects lin–12 or sel–12 activity in the same manner as sel–10. This invention also provides a method for producing suppressors of a sel–10 allele. This invenetion also provides a method for reversing the malignant phenotype of cells. This invention also provides a pharmaceutical composition effective in ameliorating Alzheimer's disease and treating cancer and methods of using such a pharmaceutical composition.

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Sel-10 AND USES THEREOF

This application is a continuation-in-part application of U.S. Serial No. 08/899,578, filed July 24, 1997, the contents of which is hereby incorporated by reference.

Part of the invention disclosed in this application was supported by the United States government, National Institute of Health grant GM 37602 and the U.S. Army Medical Research and National Command under grant DAMD 17-94-J-9410. Accordingly, the United States government has certain rights in this invention.

BACKGROUND OF THE INVENTION

Simple model organisms such as the free-living soil nematode C. elegans are experimentally tractable systems that can be used to provide insights into human development and disease. For example, genes associated with the development of cancer in humans have also been found in C. elegans. One of these human proto-oncogenes, termed *TAN-1* (Ellisen et al., 1991), encodes a protein of the LIN-12/Notch family. This family was first identified by contemporaneous studies of the *lin-12* gene in C. elegans and the *Notch* gene in Drosophila. It has been established that activating TAN-1 or a similar murine protein, Notch4, contributes to cancer formation. In C. elegans, activating LIN-12 affects cell fate decisions (Greenwald, e al., 1993; Greenwald and Seydoux, 1990; Struhl et al., 1993). Features common to all LIN-12/Notch proteins and their functions can be studied in C. elegans, and the results can be directly applied to mammals, with particular relevance to the study of cancer.

C. elegans can similarly serve as a model for processes involved in the development of Alzheimer's disease in humans. Two genes identified in linkage studies in humans encode related multipass transmembrane proteins,

presenilins 1 and 2 (PS1 and PS2). The normal role of presenilins, and the mechanism by which mutant presenilins cause Alzheimer's disease, are not known. Genetic studies of the C. elegans presenilin SEL-12 (Levitan and Greenwald, 1995; Levitan et al., 1996) offer a powerful approach to understanding the normal role of presenilins. The basic biology of presenilins and Notch proteins is linked in both C. elegans and people: based on genetic interactions with *lin-12*, *sel-12* has been shown to facilitate *lin-12* signaling in C. elegans, and null mutations in the mouse PS1 and Notch1 genes have similar phenotypes (Wong et al., 1997; Shen et al., 1997).

The following is a detailed introduction to the first set of experiments, in which the *sel-10* gene was identified as a regulator of *lin-12* activity. In the first set of experiments, genetic interactions between *sel-10* and *lin-12* were discovered, and molecular and biochemical experiments were performed to elucidate the nature of the interaction. In a second set of experiments, interactions between *sel-10* and *sel-12* were discovered, consistent with the hypothesis that *sel-10* possibly regulates *sel-12* activity.

Many cell-cell interactions that specify cell fate are mediated by receptors of the LIN-12/Notch family and ligands of the Delta/Serrate/LAG-2 (DSL) family (reviewed Artavanis-Tsakonas et al., 1995). C. elegans affords an opportunity to study a simple case of lateral specification involving an interaction between two cells of the hermaphrodite gonad. These cells, named Z1.ppp and Z4.aaa, are initially equivalent in their developmental potential: each has an equal chance of becoming the anchor cell (AC), a terminally differentiated cell type that is necessary for vulval development, or a ventral uterine precursor cell (VU), which contributes descendants to the ventral uterus. However, in any given hermaphrodite, only one of these cells will become the AC,

while the other becomes a VU (Kimble and Hirsh, 1979).

Laser ablation studies have shown that this process of lateral specification, the AC/VU decision, depends on interactions between Z1.ppp and Z4.aaa (Kimble, 1981; Seydoux and Greenwald, 1989). Furthermore, genetic studies have indicated that *lin-12*-mediated signalling controls the AC/VU decision: if *lin-12* activity is inappropriately elevated, Z1.ppp and Z4.aaa become VUs, while if *lin-12* activity is reduced, Z1.ppp and Z4.aaa become ACs (Greenwald et al., 1983). Genetic mosaic analysis (Seydoux and Greenwald, 1989) and reporter gene studies (Wilkinson et al., 1994) have indicated that both Z1.ppp and Z4.aaa initially express *lin-12* and *lag-2*, but that a stochastic small variation in ligand and/or receptor activity is subsequently amplified by a feedback mechanism that influences *lin-12* and *lag-2* transcription. Thus, Z1.ppp and Z4.aaa assess their relative levels of *lin-12* activity as part of the decision-making process, before either cell commits to the AC or VU fates, and the feedback mechanism ensures that only one of the two cells will become an AC and the other will become a VU.

It is striking that the receptors (*lin-12/Notch* proteins), ligands (DSL proteins), and at least one downstream signalling component (CBF1/Su(H)/LAG-1; see Christensen et al., 1996 and references therein) that mediate lateral specification are highly conserved in animals as distantly related as C. elegans, Drosophila, and vertebrates. Furthermore, a feedback mechanism like that first described for the AC/VU decision (Seydoux and Greenwald, 1989) also exists for a Notch-mediated lateral interaction in Drosophila (Heitzler and Simpson, 1991) and seems likely to operate in Notch-mediated lateral interactions in vertebrates (Austin et al., 1995; Chitnis et al., 1995; Washburn et al., 1997). The identification of genes that influence *lin-12* activity during the AC/VU decision may reveal other conserved factors that participate in signal

transduction or regulate the activity of *lin-12/Notch* proteins.

Genetic screens based on suppression or enhancement of *lin-12* mutations have identified a number of genes that influence *lin-12* activity. Here, *sel-10* is described. It was first identified in a screen for suppressors of phenotypes associated with partial loss of *lin-12* activity (Sundaram and Greenwald, 1993). *sel-10* acts as a negative regulator of *lin-12* signalling, and SEL-10 is a member of the CDC4 family of F box/WD40 repeat containing proteins. CDC4, the most extensively studied member of this family, is a Saccharomyces cerevisiae protein that is involved in the ubiquitin-mediated degradation of cell cycle regulators (reviewed in King et al., 1996).

The similarity of SEL-10 to CDC4 prompted investigation of the possibility that SEL-10 is involved in the ubiquitin-dependent turnover of LIN-12/Notch proteins. The experiments involved examining the biochemical effects of coexpressing C. elegans SEL-10 with a vertebrate LIN-12/Notch protein, Notch4. This vertebrate *Notch* gene was originally termed *int-3*, because it was identified by mouse mammary tumor virus insertions into a cellular gene (Gallahan and Callahan, 1989). In *int-3* mutants, the viral long terminal repeat promotes expression of a truncated transcript that encodes a protein similar to the intracellular domains of LIN-12/Notch proteins (Robbins et al., 1992). The complete sequence of the gene defined by *int3* revealed that the extracellular domain of the predicted protein also has the hallmarks of LIN-12/Notch proteins, and hence the gene is now known as *Notch4* (Uyttendaele et al., 1996). During normal development, *Notch4* expression is restricted primarily to endothelial cells (Uyttendaele et al., 1996). In *int-3* mutants, the inappropriate expression of a truncated transcript encoding the intracellular domain of Notch4 in mammary epithelia may alter stem cell fate decisions, thereby

contributing to the development of cancer. Furthermore, at least one human cancer, T cell acute lymphoblastic leukemia, has been associated with expression of a comparable truncated Notch protein (Ellisen et al., 1991), suggesting that inappropriate *Notch* activity could contribute to the development of a variety of tumors.

C. elegans SEL-10 physically interacts with murine Notch4 and causes a reduction in the steady-state levels of the murine *Notch4* intracellular domain. Results suggest that the negative regulation of LIN-12/Notch by SEL-10 is an evolutionarily conserved feature, given the striking parallels between the effect of *sel-10* activity on *lin-12* in C. elegans and the effect of SEL-10 expression on *Notch4* stability in mammalian tissue culture. Furthermore, the role of vertebrate *Notch* genes in oncogenesis suggests that vertebrate *sel-10* counterparts may behave as tumor suppressors.

SUMMARY OF THE INVENTION

This invention provides an isolated nucleic acid molecule encoding a SEL-10.

This invention also provides a purified wild-type SEL-10 protein or purified wild-type SEL-10 fragment thereof or a purified mutated SEL-10 protein or purified mutated SEL-10 fragment thereof.

This invention also provides for a method for production of an antibody capable of binding to wild-type SEL-10 or mutated SEL-10 protein comprising a) administering an amount of the purified protein or fragment of wild-type SEL-10 or mutated SEL-10 to a suitable animal effective to produce an antibody against wild-type SEL-10 or mutated SEL-10 protein in the animal; and b) testing the produced antibody for capability to bind wild-type SEL-10 or mutated SEL-10.

This invention also provides for a method for production of an antibody capable of binding to wild-type SEL-10 or mutated SEL-10 protein.

This invention further provides for an antibody capable of specifically binding to wild-type SEL-10 or mutated SEL-10.

This invention also provides a transgenic animal comprising the isolated nucleic molecule encoding SEL-10.

This invention also provides a method for identifying a compound which is capable of ameliorating Alzheimer's disease.

This invention also provides various methods for determining whether a compound is capable of ameliorating Alzheimer's disease.

This invention further provides a method for identifying a compound which is capable of treating cancer.

This invention also provides for various methods for determining whether a compound is capable of treating cancer.

This invention further provides a method for identifying a suppressor or an enhancer that affects lin-12 or sel-12 activity in the same manner as sel-10, and the suppressor or enhancer so identified.

This invention also provides a method for producing enhancers of a sel-10 allele and the enhancer so produced.

This invention also provides a method for reversing the malignant phenotype of cells.

This invention also provides a pharmaceutical composition identified as being capable of amelioraing Alzheimer's disease. This invention also provides a pharmaceutical composition identified as being capable of treating cancer and various methods of ameliorating Alzheimer's disease or treating cancer which comprises administering the above-described pharmaceutical compositions.

BRIEF DESCRIPTION OF FIGURES

Figures 1A and 1B. Molecular cloning of sel-10.
See Experimental Procedures for details of rescue assays, plasmid constructions, and molecular analysis. Genetic markers used to map sel-10 are italicized, and two cosmids that contain sel-10 sequences are shown in the box. The cosmid C07E11 and derivatives were tested for rescue. pJH169 is identical to pJH166 except that it contains a stop codon (indicated by an asterisk) in the predicted coding sequence after codon 172. Restriction sites are B, BamHI; H, HindIII; S, SalI.
1A. Restriction map of sel-10.
1B. Predicted sel-10 transcription unit.

Figures 2A-2C. cDNA sequence and predicted protein product of sel-10.
Splice junctions are indicated by arrows below the DNA sequence. The first arrow indicates the SL1 splice junction. The F-Box (Kumar and Paietta, 1995, Bai et al., 1996) is overlined and underlined and the WD40 repeats are underlined and labelled in the Figure. The lesions in sel-10(ar41) and sel-10(ar28) are indicated with bold letters in the nucleotide sequence and a bold asterisk above the amino acid; both are G to A transitions resulting in W to stop codon changes in the amino acid sequence at residues 323 and 511, respectively. The cDNA termination codon is marked with an asterisk. A sequence conforming to the consensus polyadenylation signal sequence is underlined, and sites of polyA attachment are marked in bold. Two independent cDNAs contained polyA fourteen nucleotides downstream of this signal; two alternative sites of attachment were also observed.

Figures 3A and 3B: Comparison of the sel-10, yeast CDC4 and mouse MD6 sequences.
Reverse contrast letters indicate amino acid identity between two of the three sequences. MD6 sequence

(accession number X54352) and CDC4 sequence (accession number X05625) are from Genbank.

3A. Alignment of SEL-10, CDC4, and MD6 F-Boxes.

3B. Alignment of WD40 repeats from SEL-10 and CDC4.

Figures 4A and 4B: Coimmunoprecipitation of murine Notch4(int3) and C. elegans SEL-10 from transfected 293T cells.

4A. Samples were immunoprecipitated with anti-Notch4 antibody and the Western blot was probed with anti-HA to visualize SEL-10HA (top panel) or anti-Notch4 to visualize Notch4(int3) (bottom panel).

4B. Samples were immunoprecipitated with anti-HA antibody and the Western blot was probed with anti-Notch4 (top panel) or anti-HA (bottom panel). For details see Experimental Procedures. Lane 1, mock transfected cells. Lane 2, pLNCint3 + pQNCX. Lane 3, pLNCX + pQNCsel-10HA. Lane 4, pLNCint3 + pQNCsel-10HA.

Figure 5: SEL-10 lowers the steady-state level of Notch4(int3).

Immunoblot analysis of Notch4(int3) proteins; the arrow indicates the expected mobility of Notch4(int3). Transient transfections of 293T cells were performed as described in Experimental Procedures. Lane 1, mock transfected cells (no DNA). Lane 2, pLNCint3 + pQNCX. Lane 3, pLNCint3 + pQNCsel-10.

DETAILED DESCRIPTION OF THE INVENTION

Throughout this application, references to specific nucleotides are to nucleotides present on the coding strand of the nucleic acid. The following standard abbreviations are used throughout the specification to indicate specific nucleotides:

C=cytosine	A=adenosine
T=thymidine	G=guanosine

A "gene" means a nucleic acid molecule, the sequence of which includes all the information required for the normal regulated production of a particular protein, including the structural coding sequence, promoters and enhancers.

The nucleic acids or oligonucleotides of the subject invention also include nucleic acids or oligonucleotides coding for polypeptide analogs, fragments or derivatives which differ from naturally-occurring forms in terms of the identity or location of one or more amino acid residues (deletion analogs containing less than all of the residues specified for the protein, substitution analogs wherein one or more residues specified are replaced by other residues and addition analogs where in one or more amino acid residues is added to a terminal or medial portion of the polypeptides) and which share some or all properties of naturally-occurring forms. These nucleic acids or oligonucleotides include: the incorporation of codons "preferred" for expression by selected non-mammalian or mammalian hosts; the provision of sites for cleavage by restriction endonuclease enzymes; and the provision of additional initial, terminal or intermediate DNA sequences that facilitate construction of readily expressed vectors.

The nucleic acids and oligonucleotides described and claimed herein are useful for the information which they

provide concerning the amino acid sequence of the polypeptide and as products for the large scale synthesis of the polypeptide by a variety of recombinant techniques. The molecule is useful for generating new cloning and expression vectors, transformed and transfected prokaryotic and eukaryotic host cells, and new and useful methods for cultured growth of such host cells capable of expression of the polypeptide and related products.

This invention provides for an isolated nucleic acid which encodes SEL-10. This isolated nucleic acid may be DNA or RNA, specifically cDNA, synthetic DNA or RNA, or genomic DNA. This isolated nucleic acid also encodes mutant SEL-10 or the wildtype protein. Where the isolated nucleic acid encodes a mutant SEL-10, the mutation may be generated by in vitro mutagenesis. The isolated nucleic acid molecule encoding SEL-10 may have substantially the same amino acid sequence as shown in Figure 2.

This isolated nucleic acid may also encode a polypeptide comprising the amino acid sequence of SEL-10.

As used in this application, "SEL-10" means and includes any polypeptide having SEL-10 activity, e.g. promotion of the turnover of mammalian Notch or mammalian presenilin. Thus, this term includes any such polypeptide whether naturally occurring and obtained by purification from natural sources or non-naturally occuring and obtained synthetically, e.g. by recombinant DNA procedures. Moreover, the term includes any such polypeptide whether its sequence is substantially the same as, or identical to the sequence of any mammalian homolog of the human polypeptide, e.g. murine, bovine, porcine, etc. homologs. Additionally, the term includes mutants or other variants of any of the foregoing which retain at least some of the enzymatic activity of nonmutants or nonvariants.

The invention also encompasses DNAs and cDNAs which encode

amino acid sequences which differ from SEL-10, but which do or do not produce phenotypic changes.

However, a mutant SEL-10 may not exhibit the same phenotype as the wildtype SEL-10. For example, a cell containing a mutant version of the sel-10 gene will express a protein unable to promote the degradation of mammalian Notch, or is able to better promote the degradation of mammalian Notch.

The nucleic acid of the subject invention also include nucleic acids that encode for polypeptide analogs, fragments or derivatives which differ from naturally-occurring forms in terms of the identity or location of one or more amino acid residues (including deletion analogs containing less than all of the residues specified for the protein, substitution analogs wherein one or more residues specified are replaced by other residues and addition analogs wherein one or more amino acid residues is added to a terminal or medial portion of the polypeptides) and which share some or all properties of the naturally-occuring forms.

The polypeptide of the subject invention also includes analogs, fragments or derivatives which differ from naturally-occurring forms, but retain SEL-10 activity.

This invention also provides an oligonucleotide of at least 15 nucleotides capable of specifically hybridizing with a unique sequence of nucleotides present within a nucleic acid which encodes a wildtype SEL-10 without hybridizing to a nucleic acid which encodes a mutant SEL-10. These oligonucleotides may be DNA or RNA. Such oligonucleotides may be used in accordance with well known standard methods for known purposes, for example, to detect the presence in a sample of DNA which will hybridize thereto.

As used herein, "capable of specifically hybridizing" means wherein the oligonucleotide will selectively bind to only sequences which are unique to either nucleic acids encoding wildtype or mutant SEL-10.

The oligonucleotides include, but are not limited to, oligonucleotides that hybridize to mRNA encoding SEL-10 so as to prevent translation of the protein or cause RNA-mediated interference of endogenous gene expression.

This invention also provides a nucleic acid having a sequence complementary to the sequence of the isolated nucleic acid which encodes SEL-10.

This invention also provides a vector comprising an isolated nucleic acid encoding SEL-10. The isolated nucleic acid of the vectors is operatively linked to a promoter of RNA transcription which maybe, or is identical to, a bacterial, yeast, insect or mammalian promoter. The vector may be a plasmid, cosmid, yeast artificial chromosome (YAC), bacteriophage or eukaryotic viral DNA. Specifically, this invention provides for a plasmid designated psel-10.8/1A .

This specific embodiment, psel-10.8/1A made by cleaving DNA which encodes a wildtype C. elegans SEL-10 and inserting the DNA into a plasmid. psel-10.8/1A was deposited on July 22, 1997 with the American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, Maryland 20852, U.S.A., under the provisions of the Budapest Treaty For The International Recognition Of The Deposit Of Microorganisms For The Purposes Of Patent Procedure. psel-10.8/1A has been accorded ATCC Accession Number 209154.

Further other numerous vector backbones known in the art as useful for expressing proteins may be employed. Such vectors include but are not limited to: adenovirus, simian

virus 40 (SV40), cytomegalovirus (CMV), mouse mammary tumor virus (MMTV), Moloney murine leukemia virus, murine sarcoma virus, and Rous sarcoma virus, DNA delivery systems, i.e liposomes, and expression plasmid delivery systems.

This invention also provides a vector system for the production of a polypeptide which comprises the vector in a suitable host. Suitable host includes a cell which includes, but is not limited to, prokaryotic or eukaryotic cells, e.g. bacterial cells (including gram positive cells), yeast cells, fungal cells, insect cells and animal cells.

Suitable animal cells include, but are not limited to, HeLa cells, Cos cells, CV1 cells and various primary mammalian cells. Numerous mammalian cells may be used as hosts, including, but not limited to, the mouse fibroblast cell NIH 3T3, CHO cells, Ltk$^-$ cells, etc. Expression plasmids such as that described supra may be used to transfect mammalian cells by methods well known in the art such as calcium phosphate precipitation, electroporation.

This invention also provides a method for producing a polypeptide (e.g. SEL-10) which comprises growing a host vector system under suitable conditions permitting production of the polypeptide and recovering the polypeptide so produced. Methods of recovering polypeptides produced in such host vector systems are well-known in the art and typically include steps involving cell lysis, solubilization and chromatography. This invention also provides a method of obtaining a polypeptide in purified form which comprises: (a) introducing a vector, as described above, into a suitable host cell; (b) culturing the resulting cell so as to produce the polypeptide; (c) recovering the polypeptide produced in step (b); and (d) purifying the polypeptide so recovered. As discussed above the vector may include a

plasmid, cosmid, yeast artificial chromosome, bacteriophage or eukaryotic viral DNA. Also, the host cell may be a bacterial cell (including gram positive cells), yeast cell, fungal cell, insect cell or animal cell. Suitable animals cells include, but are not limited to HeLa cells, Cos Cells, CV1 cells and various primary mammalian cells. Culturing methods useful for permitting transformed or transfected host cells to produce polypeptides are well known in the art as are the methods for recovering polypeptides from such cells and for purifying them.

Using the aforementioned method, this invention also provides a purified wildtype SEL-10 and purified fragments thereof, and a purified mutant SEL-10 and purified fragments thereof. Further, this invention also provides a polypeptide comprising the amino acid sequence of SEL-10, including, but limited to, fusion proteins having part of their amino acid sequence of the amino acid sequence of SEL-10.

Further, this invention provides where the SEL-10 produced is labeled. Different types of labeling exist. The labeling may be by various means. For instance one may tag the produced polypeptide with an established epitope such as myc. As discussed later in this application, such means of labeling are well known in the art. Further, one could also use other types of labels such as fluorescent, bioluminescent and metals.

This invention also provides a method for production of an antibody capable of binding to wild-type SEL-10 or mutated SEL-10 protein comprising: a) administering an amount of the purified protein or fragment of wild-type SEL-10 or mutated SEL-10 to a suitable animal effective to produce an antibody against wild-type SEL-10 or mutated SEL-10 protein in the animal; and b) testing the produced antibody for capability to bind wild-type SEL-10 or

mutated SEL-10.

The antibody may be produced by in vitro immunization and tested by either Western blot analysis, immunoprecipitations, or staining of cells or tissue sections.

Antibody molecules are intact immunoglobulin molecules, substantially intact immunoglobulin molecules and those portions of an immunoglobulin molecule that contains the paratope, including those portions known in the art as Fab, Fab', $F(ab')_2$ and F(v), which portions are preferred for use in the therapeutic methods described herein.

This invention also provides a method for production of an antibody capable of binding to wild-type SEL-10 or mutated SEL-10 protein comprising: a) determining conserved regions revealed by alignment of the wild-type SEL-10 or mutated SEL-10 protein sequences; b) synthesizing peptides corresponding to the revealed conserved regions; c) administering an amount of the synthesized peptides to a suitable animal effective to produce an antibody against the peptides in the animal; and d) testing the produced antibody for capability to bind wild-type SEL-10 or mutant SEL-10.

The antibody may be produced by in vitro immunization and tested by either Western blot analysis, immunoprecipitations, or staining of cells or tissue sections.

This invention also provides an antibody capable of specifically binding to wild-type SEL-10 or mutated SEL-10, and produced by the above-described methods. In a specific embodiment, the antibody is a monoclonal antibody. Further, the antibody may be labeled.

The labeled antibody may be a polyclonal or monoclonal

antibody. In one embodiment, the labeled antibody is a purified labeled antibody. The term "antibody" includes, by way of example, both naturally occurring and non-naturally occurring antibodies. Specifically, the term "antibody" includes polyclonal and monoclonal antibodies, and fragments thereof. Furthermore, the term "antibody" includes chimeric antibodies and wholly synthetic antibodies, and fragments thereof. The detectable marker may be, for example, radioactive or fluorescent. Methods of labeling antibodies are well known in the art.

This invention also provides transgenic animal comprising the isolated nucleic molecule encoding SEL-10, specifically the transgenic animal is a *Caenorhabditis elegans*.

This invention provides a method for identifying a compound which is capable of ameliorating Alzheimer's disease comprising administering an effective amount of the compound to the transgenic animal comprising the isolated nucleic acid molecule encoding SEL-10, alteration of the conditions of the transgenic animal indicating that the compound is capable of ameliorating Alzheimer's disease.

This invention also provides for a method for determining whether a compound is capable of ameliorating Alzheimer's disease comprising: a) treating *Caenorhabditis elegans* mutants having reduced, increased or altered sel-10 activity with the compound; and b) determining whether the compound suppresses, enhances or has no effect on the phenotype of the mutants, the suppression or enhancement of the phenotype indicating the compound is capable of ameliorating Alzheimer's disease. An alternative means of determination is by a) contacting the compound with a cell which expresses both SEL-12 and SEL-10; and b) determining whether the compound increases, decreases or has no effect on the amount of SEL-12 in the cell, the

increase or decrease of SEL-12 indicating that the compound is capable of ameliorating Alzheimer's disease. A third means of determination is by contacting the compound with a cell which expresses both LIN-12 and SEL-10; and determining whether the compound increases, decreases or has no effect on the amount of LIN-12 in the cell, the increase or decrease of LIN-12 indicating that the compound is capable of ameliorating Alzheimer's disease. A fourth means for determining whether a compound is capable of ameliorating Alzheimer's disease comprising: a) contacting the compound with a cell which expresses both mammalian presenilin and SEL-10; and b)determining whether the compound increases, or decreases or has no effect on the amount of mammalian presenilin in the cell, the increase or decrease of mammalian presenilin indicating that the compound is capable of ameliorating Alzheimer's disease.

As used herein "LIN-12" is a homolog of mammalian Notch protein that is found in C. elegans.

As used herein "SEL-12" is a homolog of mammalian presenilin protein that is found in C. elegans.

This invention also provides a method for identifying a compound which is capable of treating cancer comprising administering an effective amount of the compound to the transgenic animal comprising the isolated nucleic acid encoding SEL-10, alteration of the conditions of the transgenic animal indicating that the compound is capable of treating cancer.

This invention provides also a method for determining whether a compound is capable of treating cancer comprising: a) treating *Caenorhabditis elegans* mutants having reduced, increased or altered sel-10 activity with the compound; and b) determining whether the compound suppresses, enhances or has no effect on the phenotype of

the mutants, the suppression or enhancement of the phenotype indicating the compound is capable of treating cancer.

This invention also provides a method for determining whether a compound is capable of treating cancer comprising: a) contacting the compound with a cell which expresses both LIN-12 and SEL-10; and b) determining whether the compound increases, decreases or has no effect on the amount of LIN-12 in the cell, the increase or decrease of LIN-12 indicating that the compound is capable of treating cancer. Another means of determination is a) treating C. elegans mutants with the compound, b))determining whether the compound suppresses, enhances or has no effect on the phenotype of mutants, the suppression or enhancement of the phenotype indicating that the compound is capable of treating cancer. A third means of determination whether a compound is capable of treating cancer comprises a) contacting the compound with a cell which expresses both mammalian Notch and SEL-10; and b) determining whether the compound increases, decreases or has no effect on the amount of mammalian Notch in the cell, the increase or decrease of mammalian Notch indicating that the compound is capable of treating cancer.

This invention also provides a method for identifying a suppressor that affects lin-12 or sel-12 activity in the same manner as sel-10, comprising: a) mutagenizing lin-12 or sel-12 *Caenorhabditis elegans* worms with an effective amount of an appropriate mutagen: b) screening for revertants in the F1, F2, and F3 generations; and c) isolating the screened revertants, thereby identifying a suppressor of the phenotype of lin-12 or sel-12 mutation.

This invention also provides for a suppressor identified by the above-described method.

This invention also provides for a method for identifying an enhancer that affects lin-12 or sel-12 activity in the same manner as sel-10, comprising: a) mutagenizing lin-12 *Caenorhabditis elegans* worms with an effective amount of an appropriate mutagen; b) screening for enhancement in the F1, F2, and F3 generations; and c) isolating the screened enhancers, thereby identifying an enhancer of the phenotype of lin-12 or sel-12 mutation.

This invention also provides an enhancer identified by the above-described method.

This invention also provides a method for producing suppressors of a sel-10 allele comprising: a) mutagenizing sel-10 mutant hermaphrodites with an effective amount of a mutagen; b) screening for revertants in the F1, F2, and F3 generations; and c) isolating the screened revertants. This invention also provides a suppressor produced by the above-described method.

This invention also provides a method for identifying a suppressor gene comprising performing DNA sequence analysis of the suppressor identified by the above-described means to identify the suppressor gene, and the suppressor gene so identified.

This invention also provides a method for producing enhancers of a sel-10 allele comprising: a) mutagenizing sel-10 mutant hermaphrodites with an effective amount of a mutagen; b) screening for enhanced mutant in the F1, F2, and F3 generations; and c) isolating the screened enhancers. This invention also provides a enhanced mutant produced by the above-described method.

This invention also provides a method for identifying a enhancer gene comprising performing DNA sequence analysis of the enhancer identified by the above-described means to identify the enhancer gene, and the enhancer gene so

identified.

This invention also provides a method for reversing the malignant phenotype of cells comprising: a) linking the wild-type or mutated sel-10 gene to a regulatory element such that the expression of the sel-10 gene is under the control of the regulatory element; and b) introducing the linked sel-10 gene into the malignant cells for the expression of the sel-10 gene so as to reverse the malignant phenotype of cells. One could place the cells from step (b) in appropriate conditions to express the sel-10 gene such that the expression of the sel-10 gene will reverse the transforming phenotype of the malignant cells.

In the above-described method, one could induce the expression of the sel-10 gene which will reverse the transforming properties of the cells, thereby reversing the phenotype of the malignant cells in the subject.

This invention also provides for pharmaceutical compositions comprising an effective amount of the compound identified to ameliorate Alzheimer's disease and a suitable carrier.

This invention also provides a pharmaceutical compositions comprising an effective amount of the compound identified to treat cancer and a suitable carrier.

Further one could ameliorate Alzheimer's disease by administrating the above-described pharmaceutical composition in an amount effective to ameliorate Alzheimer's disease.

One could also treat cancer by administering the above-described pharmaceutical composition in an amount effective to treat cancer.

This invention is illustrated in the Experimental Details section which follows. These sections are set forth to aid in an understanding of the invention but are not intended to, and should not be construed to, limit in any way the invention as set forth in the claims which follow thereafter.

EXPERIMENTAL DETAILS

First Series Of Experiments

Experimental Procedures

1. General methods and strains

General methods are described by Brenner (1974). The wild-type parent for all strains was C. elegans var. Bristol strain N2. Mapping experiments utilized the Bristol/Bergerac congenic strain GS352, in which the region between rol-4 and par-1 of Bristol was replaced with the corresponding region from the Bergerac strain BO (Tuck and Greenwald, 1996). Strains were grown at 20° unless otherwise noted.

Mutations used are described in Hodgkin (1997); additional references for critical alleles are also given.
LGIII: dpy-17(e164), unc-36(e251) andunc-32(e189) ; lin-12(ar170) (Hubbard et al., 1996; E.J.A.H., unpublished observations); lin-12(n379) (Greenwald et al., 1983).
LGIV: dpy-20(e1282) .
LGV: nDf42 (M. Hengartner and H.R. Horvitz, personal communication), lon-3(e2175) rol-4(sc8), sel-10(ar41) (Sundaram and Greenwald, 1993), him-5(e1490), unc-76(e911).

2. Mapping of the sel-10 locus

sel-10 had been genetically mapped between lin-25 and unc-76 V (Sundaram and Greenwald, 1993), and approximately 0.2MU to the left of him-5. sel-10 was mapped between arP3 and TCPAR1 by identifying Rol Him non-Unc recombinants from heterozygotes of the genotype rol-4 BO unc-76/lon-3 sel-10 him-5 constructed using the strain GS352. Fifty independent recombinants were analyzed by

Southern blot hybridization for the presence of arP3 and TCPAR1 (see Tuck and Greenwald, 1996), and each recombinant strain was tested for the presence of sel-10(ar41) by crossing into lin-12(n379) and scoring for the Muv phenotype. Mapping data can be found in ACeDB (Edgley et al. 1997).

3. sel-10 cloning by anti-suppression assay

sel-10(ar41) partially suppresses the 2AC defect caused by lin-12(ar170): at 25°C, ~80% of lin-12(ar170) animals have 2AC while ~25% of lin-12(ar170); sel-10(ar41) animals have 2AC. Reversal of suppression was used as the basis of assessing sel-10(+) activity of microinjected DNAs. Transgenic lines were generated by microinjecting the germ lines of lin-12(ar170); dpy-20(e1282);sel-10(ar41) him-5(e1490) hermaphrodites with cosmid or plasmid DNA (Mello et al., 1991) at a concentration of 5μg/ml, along with the dpy-20(+) transformation marker DNA at 10μg/ml (plasmid pMH86; Han and Sternberg, 1991) and carrier Bluescript DNA (Stratagene) at 90μg/ml. Synchronous populations were obtained by allowing groups of transgenic hermaphrodites to lay eggs at 20°C for 1-2 hours and tranferring the eggs to 25°C. The non-Dpy L3 hermaphrodites were then scored for the number of anchor cells. The injected tester DNA was considered to contain sel-10(+) sequences if >50% of the non-Dpy animals had 2AC. Typically, 60-80% 2AC was achieved in these "rescued" lines. Arrays scored as having sel-10(+) activity were subjected to a second test, the ability to reverse the Muv phenotype of lin-12(n379);sel-10 (ar41). Initial rescue was obtained with a pool of seven overlapping cosmids from the region (each at 5μg/ml), then with the single cosmid C07E11, and then with plasmids derived from C07E11, as shown in Figure 1.

4. Plasmids containing sel-10 genomic sequences

pJH151 was constructed by digesting cosmid C07E11 with BamHI and ligating the 15kb fragment to Bluescript KS+ (Stratagene). pJH166 was constructed by ligating an 8kb PstI-SalI fragment from pJH151 into Bluescript KS+. The PstI site was from the vector while the SalI site is from the genomic sequences. The ~9kb SalI fragment was removed from pJH151 to form pJH165, and pJH167 was made by ligation of the internal HindIII fragment of pJH151 into Bluescript. To construct pJH169, pJH166 was cut with PmeI and a linker containing an NheI site with a stop codon in all frames (NEB #1060) was inserted, creating a stop codon after amino acid 172 in the SEL-10 sequence.

5. sel-10 overexpression

arEx93 was generated by microinjecting dpy-20 hermaphrodites with pJH166 [sel-10(+)] at a concentration of 100μg/ml, pMH86 [dpy-20(+)] at 10μg/ml, and Bluescript DNA at 90μg/ml. Strains carrying this array segregate sterile animals as well as fertile animals; the basis for the sterility has not been established. Many of the fertile animals display a leaky Egl phenotype similar to that observed in certain lin-12 hypomorphic mutants. Similar results were observed with other lines at this concentration and with lines established using pJH166 at 50μg/ml.

The control array arEx149 was established by microinjecting unc-32; dpy-20 hermaphrodites with pMH86 at 10μg/ml, and Bluescript DNA at 90μg/ml into unc-32; dpy-20 animals.

6. Molecular analysis of sel-10

Standard methods were used for the manipulation of recombinant DNA (Sambrook et al., 1989). sel-10(+) cDNAs were obtained by screening approximately 100,000 pfu from a phage library kindly provided by R. Barstead (Barstead and Waterston, 1989). Ten positive plaques were purified

by two subsequent rounds of screening using a radiolabelled fragment from pJH166 (~8 kb BamHI/SalI fragment) as a probe. cDNA 1A, the longest cDNA obtained, was sequenced in its entirety on one strand and compared with genomic sequence from the genome project using GENEFINDER (see Waterston et al., 1997). The sequence of the cDNA 1A differed from the GENEFINDER prediction in the location of the junction between the second and third exons and in the predicted 3' end. Four of the cDNAs were polyadenylated at their 3' ends (one 294, one 581 and the other two 601 bases after the predicted stop codon). Of these, only the last two were in the context of a conserved polyadenylation signal. The 5'-most cDNA end was located in codon 1 (cDNA 8 begins at G of the first ATG), but a PCR product was amplified from DNA prepared from the same cDNA library (Barstead and Waterston, 1989; C. Dong, personal communication) contained the SL1 spliced leader at the predicted sequence 4 bases 5' of the first ATG. The 22 base SL1 sequence and a primer straddling the 5th and 6th exons were used for the 5' end amplification.

7. Sequence analysis

Standard techniques were used to obtain sequence of the 1A cDNA (Sambrook et al., 1989). The lesions associated with the sel-10(ar41) and sel-10(ar28) mutations were found by direct sequencing of two PCR products from single-stranded templates (Allard et al., 1991, Kaltenboeck et al., 1992), using internal primers to cover the entire region. One small segment was subcloned and sequenced (from two independent reactions each), as the sequence from this region was not easily generated using the direct method. Sequence comparisons and alignments were obtained using Blast (Altschul et al., 1990) through the NCBI web site and GCG (version 8, Devereux et al., 1984) programs.

8. Plasmids for cell culture experiments

Plasmids used in the transient transfection experiments were constructed in pLNCX (Miller et al., 1989) or pQNCX (Qingyou Yan and J.K., unpublished observations), vectors that drive gene expression under the control of a CMV promoter.

pLNCint-3 contains cDNA corresponding to the Notch4 region expressed in the int3 insertion, beginning at amino acid 1411. The Notch4(int3) protein includes the entire intracellular domain of Notch4 and additional sequences (see Uyttendaele et al., 1996).

pQNCsel-10HA (pJH184) encodes a protein with a methionine-containing hemagglutinin epitope from pACT2 (Durfee et al., 1993) fused in frame (along with a short stretch of polylinker) to cDNA 1A at amino acid 13.

pQNCsel-10 contains a cDNA beginning at position +3, and encodes a protein that is probably slightly truncated at the amino terminus, most likely beginning translation at the methionine codon 9 (+27). The normal termination codon is present.

9. Transfection and Western Blot Analysis

293T cells were maintained in DMEM with 10% fetal bovine serum (FBS). A confluent plate of cells was split 1:3 the day prior to transfection. For one 60 mm plate of cells, 4 μg of each plasmid DNA was transfected using the calcium phosphate precipitation method. The total amount of DNA was kept constant by supplementation with vector DNA.

Two days after transfection, cells were harvested and lysed in TENT buffer (50mM Tris•Cl (pH8.0), 2 mM EDTA, 150 mM NaCl, 1% Triton-X 100) containing protease inhibitors (2 μg/ml aprotinin, 2 μg/ml leupeptin, 2 μg/ml pepstatin,

0.5mM PMSF). Lysates were clarified by centrifugation at 10,000g for 10 min. and protein content was determined using the BioRad Protein determination kit. Lysates containing 60μg protein were electrophoresed on 10% SDS-polyacrylamide gel and transferred onto nitrocellulose membrane. The blot was blocked overnight at 4°C with TBST(10mM Tris, pH 8.0, 150mM NaCl, 0.2% Tween 20) containing 1% Bovine serum albumin (TBST-BSA). The blot was then incubated with 1 antibody diluted (1:2,000 anti-Notch4; 1:50 for 12CA5) in TBST-BSA for 1 hour, washed three times for 5 minutes each with TBST, the blot was incubated with secondary antibody in TBST-BSA for 1 hour. After three washes, the signal was visualized by chemiluminescence (Amersham, ECL).

The anti-Notch4 antiserum (G.W. and J.K., unpublished observations) is directed against the C-terminal region of Notch4 (residues 1788-1964) (Uyttendaele et al., 1996). 12CA5 anti-HA antibody was obtained from Berkeley Antibody Co., Richmond, CA.

10. Immunoprecipitation

Subconfluent 60-mm dishes of 293T cells were calcium-phosphate transfected with 8 mg of plasmid. Two days post-transfection, cell extracts were prepared by Triton X-100 lysis, as described above, and normalized for protein content. Extracts were precleared with sepharose CL-4B beads, incubated with antibodies (3μl of anti-Notch4 antiserum or 50μl of 9E10 supernatant) for six hours at 4_C, then incubated with 40μl of 50% slurry of protein A-sepharose for 1 hour at 4°C. The protein A-sepharose beads were washed with TENT buffer three times by vortexing for 10 minutes, beads were boiled in 30μl 1X protein loading buffer, and then electrophoresed on a 10% SDS-polyacrylamide gel and subjected to immunoblot analysis, as described above.

RESULTS

Mutations that influence *lin-12* activity in C. elegans may identify conserved factors that regulate the activity of *lin-12/Notch* proteins. We describe genetic evidence indicating that *sel-10* is a negative regulator of *lin-12/Notch* mediated signalling in C. elegans are described. Sequence analysis shows that SEL-10 is a member of the CDC4 family. Biochemical data indicate that C. elegans SEL-10 physically interacts with LIN-12 and with murine Notch4, and that SEL-10 promotes LIN-12 and Notch4 degradation in mammalian cells. The parallel results obtained in C. elegans and mammalian cells suggest that negative regulation of *lin-12/Notch* activity by *sel-10* is evolutionarily conserved. We discuss potential roles are discussed for the regulation of *lin-12/Notch* activity by *sel-10* in cell fate decisions and tumorigenesis.

1. **Lowering sel-10 dosage elevates lin-12 activity**

Two *sel-10* alleles, *sel-10(ar28)* and *sel-10(ar41)*, were identified in a screen for suppressors of defects caused by a partial loss-of-function allele of *lin-12* (Sundaram and Greenwald, 1993). These *sel-10* alleles were shown to suppress multiple defects associated with loss of *lin-12* activity, and to enhance defects associated with elevated *lin-12* activity (Sundaram and Greenwald, 1993). Here, evidence is provided that shows that *sel-10* alleles reduce *sel-10* activity, indicating that *sel-10* is a negative regulator of *lin-12* activity.

For the genetic analysis of sel-10, genetic interactions with mutations in lin-12 were relied upon. Two lin-12-mediated decisions were studied (reviewed in Greenwald, 1997). One decision is made by two cells of the hermaphrodite gonad, Z1.ppp and Z4.aaa, between the anchor cell (AC) and ventral uterine precursor cell (VU)

fates; normally, only one of these two cells becomes the AC, while the other becomes a VU (see Introduction). Eliminating lin-12 activity causes both Z1.ppp and Z4.aaa to become ACs (the "2 AC defect"), and constitutively activating LIN-12 causes both Z1.ppp and Z4.aaa to become VUs. The other decision is made by the six vulval precursor cells, between a particular vulval fate termed "2°" or an alternative fate; normally, two of the six vulval precursor cells, P5.p and P7.p, adopt the 2° fate. Eliminating lin-12 activity causes all six vulval precursor cells to adopt alternative non-2° fates, and constitutively activating LIN-12 causes all six vulval precursor cells to adopt the 2° fate. Thus, mutants in which LIN-12 is constitutively active display a "0 AC Egg-laying (Egl) defect" because the absence of an AC prevents normal vulval formation; they are also Multivulva (Muv), because the descendants of each vulval precursor cell that adopts the 2° fate forms a pseudovulva.

To investigate the nature of the *sel-10(ar41)* allele, gene-dosage studies were performed assessing the enhancement of *lin-12(n379)*, a weakly activated *lin-12* allele in different sel-10 genetic backgrounds. *lin-12(n379)* homozygotes display the 0 AC-Egl defect, but do not have the Muv defect characteristic of strongly activated *lin-12* alleles (Table 1, line 4). However, double mutants display a highly penetrant 0 AC-Egl phenotype and furthermore are Muv (Table 1, lines 1 and 2 and 4 and 7), suggesting that *lin-12* activity is elevated by the *sel-10(ar41)* mutation.

Table 1.
sel-10 gene dosage analysis.

Relevant genotype	%Egl (n)	%Muv (n)	%Ste/Let (n)
20°			
lin-12(d)/+[a]	6 (93)	0 (93)	0 (93)
lin-12(d)/+;sel-10[b]	91 (54)	0 (54)	0 (54)

	15°		
lin-12(d)/+;sel-10/Df[c]	92 (39)	15 (39)	0 (39)
lin-12(d); +[d]	86 (60)	0 (60)	0 (60)
lin-12(d);sel-10/+[e]	98 (62)	0 (62)	0 (62)
lin-12(d);+/Df[f]	89 (57)[i]	62 (74)	10 (63)
lin-12(d);sel-10[g]	100 (70)[i]	78 (197)	55 (126)
lin-12(d);sel-10/Df[h]	-	85 (34)	100 (34)[j]

Complete genotypes are:

(a) *lin-12(n379)/unc-36(e251); lon-3(e2175)/him-5(e1490)*

(b) *lin-12(n379)/unc-36(e251); lon-3(e2175) sel-10(ar41)*

(c) *lin-12(n379)/unc-36(e251);lon-3(e2175) sel-10(ar41)/nDf42*

(d) *lin-12(n379); lon-3(e2175)/him-5(e1490)*

(e) *lin-12(n379); lon-3(e2175) sel-10(ar41)/him-5(e1490)*

(f) *lin-12(n379); lon-3(e2175) /nDf42*

(g) *lin-12(n379); lon-3(e2175) sel-10(ar41)*

(h) *lin-12(n379); lon-3(e2175) sel-10(ar41)/nDf42*

Complete broods were scored by picking individual L4 animals and inspecting the plates at 24 and 48 hours for the absence of eggs on the plate (Egl) and for the presence of three or more pseudovulvae along the ventral hypodermis (Muv). Plates were then inspected after an additional three days for the presence of live progeny ("Ste/Let" refers to absence of live progeny and was, in this case, a combination of sterility (Ste) and embryonic lethality(Let)). In some cases, broods were scored in batch for the Muv phenotype.

[i]Percent of fertile animals displaying the Egl defect.

[j]Inferred genotype: Complete broods from *lin-12(n379)/unc-36(e251);lon-3(e2175) sel-10(ar41)/nDf42)* were scored. The percentage of sterile non-Unc, non-Lon progeny (34/97 = 35%) is approximately equal to that expected for *lin-12(n379); lon-3(e2175) sel-10(ar41)/nDf42* genotypic class. Of the remaining 63 animals, 61/63 were unambiguously scored as heterozygotes in the next generation while the remaining 2/63 did not have a sufficient number of progeny to score unambiguously.

The enhancement of the Muv defect of lin-12(n379)/+ hermaphrodites is more pronounced when sel-10(ar41) is placed in trans to the large deficiency nDf42 (Table 1, lines 2 and 3). The greater enhancement seen in trans to

a deficiency may mean that the sel-10(ar41) allele is a partial loss of function allele rather than a null allele; alternatively, nDf42 may remove another gene that interacts with or is functionally redundant with sel-10.

Molecular data (see below) indicate that sel-10(ar41) would lead to a drastic truncation of the predicted SEL-10 protein, suggesting that sel-10(ar41) strongly reduces sel-10 activity.

Enhancement of the Muv defect of *lin-12(n379)* hermaphrodites was observed in *nDf42/+* hermaphrodites (Table 1, lines 4 and 6). This result suggests that the *sel-10* locus is haploinsufficient.

2. Elevating *sel-10* dosage lowers *lin-12* activity

The molecular cloning of *sel-10(+)* (see below) enabled examination of the effect of elevated *sel-10(+)* activity, since in general extrachromosomal arrays formed after injecting DNA at a high concentration result in higher transgene expression (Mello et al., 1991). Extrachromosomal arrays containing high-copy arrays of the *sel-10* genomic region (see below) appear to lower *lin-12* activity as assayed by their effect on the AC/VU decision. There is a dramatic decrease in the proportion of *lin-12(n379)* hermaphrodites displaying the 0 AC defect in the presence of the high copy number array *arEx93* (Table 2A). In addition, the presence of the *arEx93* array enhances the 2AC defect caused by a partial loss of *lin-12* function (Table 2B). Therefore, the level of *sel-10* activity appears to control the level of *lin-12* activity, since increasing or decreasing the activity of *sel-10* has reciprocal effects on *lin-12* activity.

Tables 2A and 2B.

Increased dosage of *sel-10* reduces *lin-12* activity.

2A) Suppression of phenotypes associated with increased *lin-12* activity

Relevant genotype	%0AC (n)
lin-12(d); dpy-20;Ex[sel-10(+) dpy-20(+)]	51 (47)
lin-12(d); dpy-20;Ex[dpy-20(+)]	95 (44)

2B) Enhancement of phenotypes associated with reduced *lin-12* activity

Relevant genotype	%2AC (n)
lin-12(h); dpy-20;Ex[sel-10(+) dpy-20(+)]	97 (34)
lin-12(h); dpy-20;Ex[dpy-20(+)]	30 (40)
lin-12(+);dpy-20(e1282);Ex[sel-10(+) dpy-20(+)]	0(0/87)

Complete genotypes: *lin-12(d)=lin-12(n379)*, *lin-12(h) = lin-12(ar170)*, *dpy-20(h) = dpy-20(e1282)*, *Ex[sel-10(+) dpy-20(+)]=arEx93*, *Ex[dpy-20(+)]=arEx149*. (See Experimental Procedures for details of strain constructions). Non-Dpy animals segregating from the strains were scored in the L3 stage for the number of anchor cells.

3. *Sel-10* Mutants Display Low Penetrance Defects Associated with Constitutive Activation of *Lin-12*

Most *sel-10* animals appear wild-type. About 1% of *sel-10(ar41) him-5* hermaphrodites were observed to lack an AC. Furthermore, about 4% of *sel-10(ar41)* males display a gonad Migration (Mig) defect similar to that seen in *lin-12(d)* mutants, where it results from to failure to form the linker cell, the male counterpart of the hermaphrodite AC (Greenwald et al., 1983). In addition, about 8% of *sel-10* mutant hermaphrodites are Egl even though they have an AC, and that *sel-10* males have a reduced mating efficiency that can not be completely accounted for by the Mig defect. These additional defects may reflect the effect of increased *lin-12* activity on other cell fate decisions (Greenwald et al., 1983).

4. Cell Autonomy of Sel-10 Function

Two lines of evidence suggest that *sel-10* functions cell autonomously to elevate *lin-12* activity. First, the

effect of reducing *sel-10* activity on the activity of the intracellular domain of LIN-12 was examined. Expression of *lin-12(intra)* causes phenotypes associated with LIN-12 activation (Struhl et al., 1993). Since LIN-12(intra) lacks the extracellular domain and hence is active in the absence of external signalling, an enhancement of *lin-12(intra)* activity by *sel-10* mutations would be evidence for cell autonomy of *sel-10* function. An extrachromosomal array that contains the *lin-12(intra)* transgene and a transformation marker were used (see Experimental Procedures); this array results in a low-penetrance *lin-12* activated phenotype (Table 3A).

Tables 3A and 3B.

Cell autonomy of *sel-10* function.

3A) Enhancement of *lin-12*(intra)

Relevant genotype	%Egl (n)	%Mig (n)
sel-10(+);arEx[lin-12(intra)]	16 (88)[a]	10 (57)
sel-10(ar41);arEx[lin-12(intra)]	46 (136)[a]	59 (90)

All strains also contained *him-5(e1490)*. *arEx[lin-12(intra)]* = *arEx152* (Fitzgerald, personal communication) is an extrachromosomal array formed by microinjection (Fire, 1986; Mello et al., 1991) of pRF4 (plasmid containing *rol-6(su1006)* sequence that confers a Rol phenotype onto worms carrying the array) at 100µg/ml and pLC8 (Struhl et al., 1993).

[a]We infer that these Egl hermaphrodites lacked an AC because we scored additional hermaphrodites of relevant genotype *sel-10; arEx[lin-12(intra)]* in the L3 stage for the presence or absence of an AC and as adults for their egg-laying ability, and found that nine hermaphrodites that clearly had a single AC were non-Egl, while nine hermaphrodites that clearly lacked an AC were Egl.

3B) Cell Ablation

Relevant genotype	%0AC (n) unoperated	%0AC (n) operated
lin-12(n379)/+ ; sel-10(+)	10 (57)	9 (11)
lin-12(n379)/+; sel-10(ar41)	97 (71)	83 (12)

Complete genotype: *dpy-17(e164) lin-12(n379)/unc-32(e189); lon-3(e2175) sel-10(+ or ar41)*

"Operated" refers to worms in which Z4 was laser ablated in the early L1 stage (when the gonad primordium consisted of four cells, Z1-Z4). Worms were then scored in the L3 stage for the presence or absence of an AC.

When this array is combined with *sel-10(ar41)*, there is a dramatic increase in the proportion of hermaphrodites displaying the 0AC-Egl defect and males displaying the Mig defect (Table 3A), suggesting that *sel-10* (+) activity normally reduces lin-12 function in the same cell.

sel-10 functions were tested in the receiving end of *lin-12*-mediated cell-cell interactions by performing cell ablation experiments to remove the signalling cell, in this case Z4.aaa (Table 3B). This experiment enables different genotypes to be compared with respect to their intrinsic level of constitutive *lin-12* activity in Z1.ppp.

If Z4, the precursor to Z4.aaa, is ablated in *lin-12(n379)/+* hermaphrodites, Z1.ppp usually becomes an AC, because the level of constitutive *lin-12* activity is relatively low. However, if Z4 is ablated in *lin-12(n379)/+; sel-10* hermaphrodites, Z1.ppp usually becomes a VU, suggesting that the level of constitutive *lin-12* activity is relatively high. These results suggest that *sel-10* (+) functions to reduce *lin-12* activity cell autonomously, since a high level of intrinsic *lin-12* activity is seen when *sel-10* activity is reduced even when the signalling cell is removed.

5. Cloning of Sel-10 by an Anti-Suppression Assay

sel-10 was previously mapped to an interval between *lin-25* and *unc-76* on LGV(Sundaram and Greenwald, 1993). The map position to a 300 kb interval between the cloned polymorphisms *arP3* and *TCPAR1* space were refined (see Experimental Procedures and Fig. 1). Cosmids from the region were tested for their ability to reverse the

suppression of the 2AC defect of *lin-12(ar170)* by *sel-10(ar41)* (see Experimental Procedures). The cosmid C07E11 gave rescue in this anti-suppression assay and also reversed the enhancement of *lin-12(n379)* by *sel-10(ar41)*. This cosmid was further subcloned and the ~8kb fragment in pJH166 gave results similar to that seen with the entire cosmid (Figure 1).

6. Molecular Analysis of *Sel-10*

The ends of pJH166 (Figure 1) were sequenced and compared with sequence generated by the C.elegans genome sequencing project (Waterson et al., 1997). The entire region was found on the cosmid F55B12. A fragment from the predicted open reading frame (genefinder) was radiolabeled and used to screen a Northern blot and to probe a cDNA library (see Experimental Procedures). Northern analysis revealed a single band of ~2.5kb which is present in total RNA prepared from wild type, *sel-10(ar41)* and *sel-10(ar28)* strains. The ends of ten cDNAs were sequenced and the largest cDNA was sequenced in its entirety on one strand. Verification of the 5′ end was obtained by sequencing products amplified from the cDNA library using the trans spliced leader sequence SL1 (Krause and Hirsh, 1987) and a *sel-10*-specific sequence for primers. The splice junction of SL1 to the *sel-10* coding region occurs four bases upstream of the first predicted start codon. Figure 2 summarizes the results of the sequence analysis of sel-10.

7. Sel-10 Encodes a Protein of the Cdc4 Family of Proteins

A BLAST search (Altschul et al., 1990) using the predicted SEL-10 protein sequence revealed that it contains two previously identified amino acid sequence motifs (Figure 3). There are seven tandem WD40 repeats, also known as βtransducin repeats, a conserved repeat of approximately

40 amino acids named for the common appearance of Trp-Asp (WD) at the end of the repeat (reviewed in Neer et al., 1994). The crystal structure of βtransducin reveals that the seven repeats form a β propeller structure, which most likely mediates protein-protein interactions (Gaudet et al., 1996; Lambright et al., 1996; Sondek et al., 1996). There is a great deal of functional diversity among WD40 repeat-containing proteins.

There is another motif (Kumar and Paitta, 1995) that is now called the F-Box, after its occurrence in cyclin F (Bai et al. 1996). The F-Box motif has also been implicated in protein-protein interactions, and is found in a large variety of proteins, many of which contain other recognizable motifs C-terminal to the F-Box (Bai et al., 1996).

The presence of an F box N-terminal to a set of seven WD40 motifs is the hallmark of the CDC4 family of WD40-repeat containing proteins, so SEL-10 appears to belong to this family. Furthermore, separate BLAST searches with just the SEL-10 WD40 repeats or the SEL-10 F box always identified members of the CDC4 family as the most similar. The alignment of the WD40 repeats of SEL-10 and CDC4 (Fig. 3B) reveals that a given WD40 repeat is more similar between yeast and worms than are the repeats within a given species. In addition, the F-Box motif present in proteins within the CDC4 subfamily is more conserved than among other F-Box-containing proteins (Figure 3A), and there is more extensive homology around the F-box (Kumar and Paitta, 1995).

The CDC4 family includes proteins in fungi and vertebrates, as well as two other predicted C. elegans proteins (see Discussion). The best studied member of this family, S. cerivisiae CDC4 targets Sic1 and certain G1 cyclins for degradation (reviewed in King et al., 1996). However, not all CDC4 family members are cell

cycle regulators. For example, there are proteins that negatively regulate sulfur metabolism from *S. cerevisiae*, *Neurospora crassa* and *Aspergillis nidulans* (Natorff et al., 1993; Kumar and Paietta, 1995; Thomas et al., 1995). Biochemical mechanisms for other CDC4 family members have not been described.

8. Sel-10 Mutations Truncate the SEL-10 Protein

Sequence analysis of *sel-10* mutations supports the genetic evidence suggesting that they strongly reduce *sel-10* activity. The sequence alterations caused by *sel-10(ar41)* and *sel-10(ar28)* were determined by direct sequencing of amplified genomic DNA products (see Experimental Procedures). Both alleles are nonsense mutations at nucleotide positions 969 and 1533, respectively (see Fig. 2), resulting in truncated predicted proteins.

Sel-10(ar41) removes the C terminal half of the protein, including five of the seven WD40 repeats. This observation suggests that *sel-10(ar41)* is likely to result in a nonfunctional SEL-10 protein. It is unlikely that the two WD40 repeats that remain in this protein are functional since there are no known WD40-repeat containing proteins with only two repeats (Neer et al.,1994). Furthermore, the crystal structure of β transducin reveals that the seven repeats form a β propeller structure that would not be complete in the absence of five of the seven repeats (Sondek, et al., 1996). Finally, comparable mutations in another C. elegans CDC4 subfamily protein, LIN-23, behave like molecular null alleles (Kipreos, et al., submitted).

9. C. elegans SEL-10 Physically Interacts with LIN-12(intra) and Murine Notch4(int3)

The observations that *sel-10* negatively regulates *lin-12* activity and resembles CDC4 suggested the possibility that

SEL-10 functions biochemically like CDC4 to promote LIN-12 turnover. This model makes two predictions. First, SEL-10 should physically interact with LIN-12/Notch proteins. Second, the steady-state level of LIN-12/Notch proteins should be reduced by expression of SEL-10.

Potential interactions between SEL-10 and the intracellular domains of LIN-12/Notch proteins were probed, specifically LIN-12(intra), the intact intracellular domain (see Struhl et al., 1993) and Notch4(int3), the intact intracellular domain with some additional sequences produced by the int3 mutation (Robbins et al., 1992; Uyttendaele et al., 1996). Initially the yeast two-hybrid system (Fields and Song, 1989) was used and preliminary results suggested that SEL-10 physically interacted with the C. elegans LIN-12 and GLP-1 intracellular domains, and the mouse Notch4(int3) intracellular domain. To examine further whether SEL-10 binds LIN-12/Notch proteins in vivo, co-immunoprecipitation experiments using transfected mammalian cells were carried out (Fig. 4). 293T (Bosc) cells (human embryonic kidney cells) were transiently transfected with Notch4(int3) and/or HA-tagged SEL-10. Transfected cells were lysed and Notch4(int-3) was precipitated with anti-Notch4 antibodies or, alternatively, SEL-10HA was immunoprecipitated with anti-HA antibodies. The immunoprecipitates were subjected to immunoblot analysis to identify bound proteins, and probed with anti-HA and anti-Notch4 antibodies. Under both conditions, the immunoprecipitates were found to contain both Notch4(int3) and SEL-10HA. Similar results were obtained using Myc-tagged SEL-10. It was concluded that SEL-10 can be found in a complex with Notch4 in vivo.

Similar experiments were performed with epitope-tagged LIN-12(intra), and the coimmunoprecipitation results suggested that LIN-12 and SEL-10 also physically interact

(data not shown). However, LIN-12(intra) was poorly expressed in mammalian cells, so we proceeded with our analysis using Notch4(int3) exclusively.

10. C. elegans SEL-10 Reduces the Steady State Levels of Murine Notch4(int3)

If SEL-10 functions similarly to CDC4 in targeting specific proteins for proteolysis, then expression of SEL-10 might reduce the level of LIN-12/Notch proteins. The effect of expressing SEL-10 on the steady state levels of Notch4(int3) was examined.

Transient transfection experiments in 293T cells were performed, in which the steady state level of Notch4(int3) was examined in the presence or absence of SEL-10. Western blot analysis revealed that the steady state level of Notch4(int3) is reduced in cells expressing SEL-10 as compared to mock transfected cells (Fig. 5a). Three independent transient transfection experiments performed in this manner yielded comparable results. A reduction in the steady state level of Notch4(int3) when epitope-tagged forms of SEL-10 were used in transient transfection experiments were also usually observed. In the co-immunoprecipitation experiments described above, less Notch4(int3) precipitated from cells were consistently seen that also contained transfected SEL-10, consistent with a reduced steady-state level of Notch4(int3) protein in the presence of SEL-10 (see Fig. 4).

The results suggest that the biochemical mechanism by which sel-10 functions as a negative regulator of lin-12/Notch activity is by promoting LIN-12/Notch degradation. Applicants were unable to look directly at whether SEL-10 promotes ubiquitination of Notch4(int3) because there is substantial polyubiquitination of Notch4(int3) even in the absence of transfected SEL-10.

The fact that the effects we see in this transfection system are relatively modest, and that ubiquitination occurs even in the absence of transfected SEL-10, may be due to the activity of an endogenous murine sel-10-like gene.

Discussion

In this paper, genetic evidence indicates that *sel-10* is a negative regulator of *lin-12* mediated signalling in C. elegans. Mutations that lower *sel-10* activity elevate *lin-12* activity, and increasing *sel-10* dosage lowers *lin-12* activity. Furthermore, *sel-10* appears to act in the same cell as *lin-12*.

Molecular and biochemical evidence suggest that the mechanism by which *sel-10* controls *lin-12* activity is by controlling LIN-12/Notch protein levels. First, sequence analysis indicates that SEL-10 is related to the Saccharomyces cerevisiae protein CDC4, which is involved in the ubiquitin-dependent degradation of cell cycle regulators (reviewed in King et al., 1996). Second, C. elegans SEL-10 physically interacts with the intracellular domain of LIN-12 and with Notch4(int3), the intracellular domain of murine Notch4. Third, coexpression of SEL-10 with Notch4(int3) causes a reduction in the steady-state level of Notch4(int3).

The effects of *sel-10* activity on *lin-12* in C. elegans and the effect of SEL-10 expression on Notch4 stability in culture are strikingly parallel. Furthermore, C. elegans SEL-10 interacts physically with murine Notch 4, and proteins related to SEL-10 exist in mammals. These observations suggest that the negative regulation of LIN-12/Notch by SEL-10 is an evolutionarily conserved feature. Evidence suggests that SEL-10 targets LIN-12/Notch proteins for degradation. Also, potential roles for protein turnover in LIN-12/Notch-mediated cell

fate decisions were considered.

1. SEL-10 May Target LIN-12/Notch Proteins for Ubiquitin-Mediated Degradation

The attachment of ubiquitin to substrates involved a series of protein complexes. Ubiquitin is activated by linkage to an E1 ubiquitin activating enzyme, then transferred to an E2 ubiquitin conjugating enzyme. Some ubiquitination events also require the action of a third complex, termed E3. It is thought that E3 complexes may contribute to substrate specificity (reviewed in Ciechanover, 1994; King et al., 1996). The Saccharomyces cerevisiae protein Cdc4p may function in an E3 complex. *CDC4* is one of a group of genes that also includes *CDC34*, *CDC53*, and *SKP1*; together, these genes directly regulate the level of the cyclin dependent kinase inhibitor Sic1p, which must be destroyed for progression from G1 to S phase. Cdc34p is an E2 ubiquitin conjugating enzyme (Goebl et al., 1988), and the current view is that Cdc4p, Cdc53p, and Skp1p function as an E3 complex (Bai et al., 1996; Mathias et al., 1996). Based on analysis of *sel-10* and the data for CDC4, SEL-10 may function as part of an E3 complex to target the intracellular domains of LIN-12/Notch proteins for ubiquitin-dependent degradation.

An important issue to consider in the context of SEL-10 as a component of an E3 complex is its specificity for LIN-12/Notch proteins. The available C. elegans genetic data suggest that *sel-10* an allele-nonspecific, gene-specific suppressor, supporting a role for SEL-10 specifically in regulating the activity of LIN-12, or perhaps a small subset of proteins that includes LIN-12. Allele-nonspecificity is indicated by the observation that mutations in *sel-10* suppress/enhance all *lin-12* alleles tested (Sundaram and Greenwald, 1993b; this work; E.J.A.H., unpublished observations). Gene-specificity is

suggested by the fact that mutations in *sel-10* have not been identified in numerous screens in many laboratories for suppressors of other hypomorphic mutations; furthermore, suppression of various marker mutations used in routine strain constructions of hypomorphic alleles of several other genes encoding receptor proteins (E.J.A.H., unpublished observations).

The available genetic data also suggest that *sel-10* activity is not necessary for cell cycle progression, a possibility raised by the phenotype of *cdc4* mutants. Mutations in cul-1, a C. elegans gene related to S. cerevisiae CDC53, cause hyperplasia of larval blast cells, suggesting that cul-1 regulates cell-cycle progression (see Kipreos et al., 1996). No evidence that hyperplasia occurs in *sel-10(ar41)* mutants has been seen. (E.J.A.H., unpublished observations). Since *sel-10(ar41)* mutants have little or no *sel-10* activity (see Results), it is unlikely that *sel-10* is involved in cell cycle regulation per se, unless there is another functionally redundant gene that masks cell cycle involvement of *sel-10*. In contrast, mutations in another CDC4 related gene, *lin-23*, do cause hyperplasia, consistent with a role for *lin-23* in the regulation of cell cycle progression (Kipreos et al., submitted).

2. SEL-10 Mediated Degradation of LIN-12/Notch as a Mechanism for Receptor Downregulation

For a variety of cell surface receptors, ligand-induced polyubiquitination is thought to be a mechanism for down-regulation (reviewed in Ciechanover and Schwartz, 1994). It is proposed that ubiquitin-mediated degradation is also a mechanism for down-regulation of activated LIN-12/Notch proteins, and that SEL-10 plays a critical role in this process. Although no direct evidence exists for ligand-induced ubiquitination of LIN-12/Notch receptors, the free and intact intracellular domains of

LIN-12/Notch proteins have been shown to behave like activated receptors (Lieber et al., 1993; Struhl et al., 1993), and that Notch4(int3) behaves like a gain-of-function mutation (Gallahan and Callahan, 1987). Biochemical data are therefore consistent with the proposal that the activated receptor may be the substrate for SEL-10-mediated polyubiquitination. For the remainder of the Discussion, possible roles of SEL-10 mediated down-regulation of LIN-12/Notch proteins in cell fate decisions and oncogenesis are considered.

3. Potential roles for SEL-10 mediated LIN-12 down-regulation in the AC/VU decision and VPC specification

sel-10 influences lin-12 activity in the AC/VU decision. The AC/VU decision can be considered to involve three phases prior to the committed state (Seydoux and Greenwald, 1989; Wilkinson et al., 1994). Initially, Z1.ppp and Z4.aaa have equal signalling and receiving potentials; ligand (LAG-2) and receptor (LIN-12) may interact, but signalling activity is below a critical threshold. A small stochastic difference between the two cells, such that one cell has a greater level of signalling activity and the other has a greater level of receiving activity, is amplified by a feedback mechanism that involves differential transcription of ligand and receptor genes. This feedback mechanism amplifies the stochastic difference that has arisen between the two cells and ensures that the cell in which *lin-12* activity is greater becomes the VU while the cell in which *lin-12* activity is less becomes the AC.

The ultimate consequences of LIN-12 activation must be on gene expression. For the feedback mechanism, this effect has been visualized for the transcription of *lag-2* and *lin-12*: activation of LIN-12 appears to repress transcription of *lag-2* and stimulate transcription of

lin-12 (Wilkinson et al., 1994). At a minimum, downstream genes needed for differentiation as a VU must be activated; it is also likely that genes involved in the differentiation of an AC are repressed. *sel-10* might influence the AC/VU decision because, unless LIN-12 is down-regulated, the initial signalling that occurs between Z1.ppp and Z4.aaa might cause both cells to achieve the threshold value of effector activity.

Most *sel-10(ar41)* individuals are phenotypically wild-type, with only a small proportion displaying phenotypes associated with LIN-12 activation, may be explained in this context if there is a redundant gene product or regulatory mechanism. There is at least one additional CDC4 related gene in the C. elegans genome (E.J.A.H., unpublished observations). Furthermore, there may be other mechanisms for degrading activated LIN-12. For example, *sel-1*, another negative regulator of *lin-12* activity, may also be involved in LIN-12 turnover (see Grant and Greenwald, 1997), but since SEL-1 is a secreted or extracytosolic membrane-associated protein found in intracellular vesicles, it is not likely to be directly involved in the ubiquitination of the intracellular domain of LIN-12.

4. Potential Roles for Degradation of Activated LIN-12/Notch Proteins in Other Cell Fate Decisions

In other cell fate decisions, including specification of the vulval precursor cells (VPCs), the role of sel-10 in the VPC decions may be similar to its role in the AC/VU decision: to ensure that a threshold value of LIN-12 activation must be reached in the appropriate cells for commitment. However, regulated turnover of activated receptors may play different or additional roles in other LIN-12/Notch mediated decisions. For example, in Drosophila eye development, *Notch* appears to be utilized for sequential cell fate decisions (Cagan and Ready,

1989), which would seem to necessitate clearance of activated *Notch* after each decision so that a new assessment of *Notch* activity can be made. Furthermore, it is also conceivable that for some LIN-12/Notch mediated decisions, the cell fate adopted may depend on the intensity of signal, as has been seen for receptors for gradient morphogens (e.g., Nellen et al., 1996). If any LIN-12/Notch-mediated decisions do display such dosage sensitivity, it is likely that they would depend on rapid turnover of activated receptor complexes so that the correct threshold value is read.

5. Potential Roles for *sel-10* in Oncogenesis

When the intact intracellular domain of LIN-12/Notch proteins is expressed, cell fate transformations known to be associated with activation of LIN-12/Notch proteins are seen, indicating that the intact intracellular domain behaves like a constitutively active receptor (Lieber et al., 1993; Struhl et al., 1993). Thus, the observation that mammalian tumors can be induced by expression of truncated forms of Notch largely consisting of the intact intracellular domain (Ellisen et al., 1991; Robbins et al., 1992; Uyttendaele et al., 1996) suggests that constitutive *Notch* activity can be a causal factor in tumor formation. Since SEL-10 downregulates *Notch* activity, it may act to restrain the either normal or oncogenic functions of activated *Notch*, and hence suppress cell growth. If so, then loss-of-function mutations in vertebrate *sel-10* could contribute to tumor formation by elevating the level of *Notch* protein. For instance, human T acute lymphoblastic leukemias, which in the majority of cases do not contain oncogenic *Notch* alterations (Drexler et al., 1995), and human breast tumors, which thus far have not been reported to contain oncogenic *Notch* alterations, may carry mutations in other proteins that influence Notch activity, such as *sel-10* homologs.

Second Series of Experiments

1. Genetic Interaction Between *sel-10* and *sel-12*

sel-12(ar171) and *sel-12(ar131)* cause an egg-laying defective (Egl) phenotype. *sel-10(ar41)* suppresses the Egl defect of *sel-12* mutants (Table 4).

Table 4. All strains also contained the markers him-5(e1490), and unc-1(e538).

Relevant genotype	#Egl$^+$/total (%)	
sel-10(+); sel-12(ar131)	2/99	(2%)
sel-10(ar41); sel-12(ar131)	88/118	(75%)
sel-10(+); sel-12(ar171)	0/107	(0%)
sel-10(ar41) sel-12(ar171)	25/126	(20%)

2. Potential physical interaction between SEL-10 and SEL-12, and SEL-10 and human presenilin 1 (PS1)

The genetic interaction between *sel-10* and *sel-12* raises the possibility that there is a direct physical interaction between the SEL-10 and SEL-12 proteins that promotes the ubiquitin-mediated turnover of SEL-12 presenilin. Experiments similar to those described in the first series of experiments, substituting SEL-12 in place of LIN-12, can be performed. Specifically, co-immunoprecipitation of epitope-tagged forms of SEL-10 and SEL-12, can be performed lowering of steady-state levels of SEL-12 in the presence of SEL-10, and ubiquitination of SEL-12 in the presence of SEL-10 will be observed. Similar experiments with human PS1, other human presenilins (e.g. PS2) and other C. elegans presenilins (HOP-1) can be performed. The genetic interactions of SEL-10 and other C. elegans presenilins will also be performed.

3. Implications for Alzheimer's disease

If there is a physical interaction between SEL-10 and SEL-12, such that SEL-10 promotes the ubiquitin-mediated degradation of SEL-12, then compounds that interfere with this process will be potential drugs for Alzheimer's disease.

If there is no direct physical interaction between SEL-10 and SEL-12, then the suppression of *sel-12* mutations by *sel-10* mutations is indirect, perhaps through effects on LIN-12. However, given the intimate connection between LIN-12/Notch and SEL-12/presenilin in C. elegans (Levitan and Greenwald, 1995) and mice (Wong et al., 1997; Shen et al., 1997), then compounds that interfere with the degradation of LIN-12 will be potential drugs for Alzheimer's disease.

4. *sel-10* as a starting point for screens for other potential targets

A) Yeast two-hybrid screen for proteins that interact with SEL-10.

The two-hybrid screen, originally developed by Fields and Song (1989), is a powerful strategy for identifying potential interacting proteins. The screen relies on the ability of GAL4 to activate transcription of a reporter gene containing GAL4 upstream activation sequences. GAL 4 has a DNA binding domain (GBD) and a activation domain (GAD). Normally, the two domains are present in the same polypeptide; if they are separated, GAL4 activity is abolished. However, if the separated domains are joined to protein sequences that interact with each other, the two domains are brought together, and GAL4 activity is restored. Thus, a yeast strain containing a "bait" fused to the GBD is transformed with a library containing potential GAD fusions, and a selection or screen for

reconstituted GAL4 activity is used to identify candidates.

This method to identify genes that interact with *lin-12* (Hubbard et al., 1996) has been used. A similar approach to identifying genes that interact with *sel-10* will be used. A bait containing sequences from SEL-10 fused to GBD has been prepared, and a derivative of yeast strain Y153 with this plasmid will be prepared. Y153 contains GAL4 UAS--HIS3 and GAL 4 UAS--lacZ, enabling candidates to be selected by the ability to grow on medium containing 3AT (HIS+ selection) or screened for beta-galactosidase activity (Durfee et al., 1993). Clones of the excellent random oligomer-primed library prepared by Bob Barstead by transforming the yeast strain Y153 and selecting for growth on 3AT plates will be screened; these transformants for beta-galactosidase activity will be screened in a filter assay. Then, from each candidate, the candidate worm-GAD plasmid will be isolated and retested with the bait (SEL-10-GBD), and with SNF1-GBD and/or lamin-GBD to check for general stickiness.

Any candidates that specifically bind SEL-10-GBD will be isolated and sequenced. The sequence analysis and derived map position (using the genome project database) may help illuminate their functions. To see if the physical interaction is functionally relevant in vivo, C. elegans mutants will be created by RNA interference or gene-knockout strategies using established methods. The candidate mutants for phenotypes and for genetic interactions with existing *sel-12*, *sel-10*, and *lin-12* mutants will then be examined.

B) Genetic screens for mutations that interact with *sel-10* will be conducted, specifically looking for extragenic suppressors or enhancers.

Screens that may be used are:

1) Mutagenizing appropriate strains so as to generate hermaphrodites of relevant genotype *lin-12(n379); sel-10/Df(sel-10); ** [where * represents a mutagenized chromosome] and look for fertile hermaphrodites. These individuals may carry extragenic suppressors that define new components of the *sel-10* pathway or complex. Genetic and molecular characterization of extragenic suppressors of *sel-10*, including their interaction with mutations in *sel-12* and *lin-12*, may identify other potential targets for drugs against Alzheimer's disease and cancer, other potential tumor suppressors or oncogenes that act in the *lin-12* pathway, and other potential genes influencing the development of Alzheimer's disease.

2) Mutagenizing appropriate strains so as to generate hermaphrodites of relevant genotype *arEx93 [sel-10(+)]; ** to revert the sterility defect associated with *sel-10* overexpression. Other high copy number or highly expressing arrays in place of *arEx93* may also be used. As described above, these individuals may carry extragenic suppressors that define new components of the *sel-10* pathway or complex. Genetic and molecular characterization of extragenic suppressors of *sel-10*, including their interaction with mutations in *sel-12* and *lin-12*, may identify other potential targets for drugs against Alzheimer's disease and cancer, other potential tumor suppressors or oncogenes that act in the *lin-12* pathway, and other potential genes influencing the development of Alzheimer's disease.

Third Series of Experiments

Mutations in either of two presenilin genes (*PS1* and *PS2*) cause Alzheimer's disease, and reducing presenilin level is a potential therapeutic target for treating Alzheimer's disease (DeStrooper, *et al.*, 1998). We have found genetic and physical interactions between *C. elegans* SEL-10, a member of the CDC4 family of proteins (Hubbard, *et al.*, 1997), and SEL-12, a *C. elegans* presenilin (Levitan and Greenwald, 1995). We show that a loss of *sel-10* activity can suppress the phenotype associated with reducing *sel-12* activity and that SEL-10 can physically complex with SEL-12. Proteins of the CDC4 family have been shown to target proteins for ubiquitin-mediated turnover (Hoyt, 1997). The functional and physical interaction between *sel-10* and *sel-12* therefore offers an approach to understanding how presenilin levels are normally regulated, and therefore how they may be reduced.

sel-12 mutations cause a highly penetrant egg-laying defective (Egl) phenotype (Levitan & Greenwald, 1995). SEL-12 is 50% identical to human PS1 and PS2 and appears to be a bona fide presenilin, since either human PS1 or PS2 can efficiently substitute for SEL-12 in *C. elegans* (Levitan, *et al.*, 1996). Another *C. elegans* gene, *hop-1*, encodes a somewhat more divergent presenilin (Li & Greenwald, 1997). In principle, loss-os-function mutations that suppress the Egl defect of *sel-12* mutants might augment or stabilize mutant SEL-12 proteins or HOP-1(+), or bypass the need for presenilin activity.

sel-10(ar41) is a strong loss-of-function mutation that dramatically suppresses the Egl defect caused by *sel-12(ar131)* or *sel-12(ar171)* (Table 1). *sel-10(ar41)* also suppresses certain cell fate transformations associated with reducing the activity of *lin-12*, which encodes a receptor of the LIN-12/Notch family (Hubbard, *et al.* 1997), (Sundaram & Greenwald, 1993). However, the

suppression of the *sel-12* Egl defect by reducing *sel-10* activity is unlikely to be simply an indirect consequence of the effect of *sel-10* on *lin-12* activity, since *sel-10(ar41)* efficiently suppresses *sel-12* mutations (Table 1) but is unable to suppress the Egl defect caused by reducing *lin-12* activity (Hubbard, *et al.* 1997), (Sundaram & Greenwald, 1993).

If presenilins are indeed a target of SEL-10, then SEL-10 would be expected to associate physically with presenilins. Potential physical interactions were assessed between SEL-10 and SEL-12 by co-immunoprecipitation using transiently transfected mammalian cells. Cells were cotransfected with expression plasmids encoding myc-tagged SEL-10 and HA-tagged SEL-12. Transfected cells were lysed and cell lysates were precipitated with either anti-myc antibodies or anti-HA antibodies. The anti-myc immunoprecipitate was found to contain both SEL-10myc and SEL-12HA. This result suggests that SEL-10 and SEL-12 presenilin are able to form a complex.

Database analysis reveals that proteins that are highly related to SEL-10 exist in mammals (Hubbard, *et al.*, 1997). The functional and physical interactions between SEL-10 and SEL-12 raise the intriguing possibility that SEL-10 is part of a mechanism that regulates presenilin level or activity in mammals. Manipulation of *sel-10* activity, as well as knowledge from further studies of the nature of *sel-10* interaction with *sel-12*, may lead to therapeutic strategies for ameliorating Alzheimer's disease.

Table 1. Reducing *sel-10* activity suppresses the egg-laying defect (Egl) of *sel-12* mutants. All strains also contained the markers *him-5(e1467)* and *unc-1(e538)* to facilitate genetic manipulations.

Relevant Genotype	#non-Egl/total(%)
sel-10(+);sel-12(ar131)	2/99 (2%)
sel-10(ar41);sel-12(ar-131)	88/118 (75%)
sel-10(+);sel-(12ar171)	0/107 (0%)
sel-10(ar41);sel-12(ar171)	25/126(20%)

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What is claimed is:

1. An isolated nucleic acid molecule encoding a SEL-10.

2. The isolated nucleic acid molecule of claim 1, which encodes a mutated SEL-10.

3. The isolated nucleic acid molecule of claim 2, wherein the mutation is generated by in vitro mutagenesis.

4. The isolated nucleic acid molecule of claim 1, 2 or 3, wherein the molecule is a DNA, cDNA, genomic DNA, synthetic DNA or RNA.

5. The isolated nucleic acid molecule of claim 1, wherein the encoded SEL-10 has substantially the same amino acid sequence as shown in Figure 2.

6. A nucleic acid molecule of at least 15 nucleotides capable of specifically hybridizing with the sequence of a nucleic acid molecule of claim 1.

7. The nucleic acid molecule of claim 6, wherein the molecule is DNA or RNA.

8. An isolated nucleic acid molecule of claim 1 operatively linked to a promoter of RNA transcription.

9. 8A vector which comprises the isolated nucleic acid molecule of claim 1.

10. The vector of claim 9, wherein the vector is a plasmid.

11. The plasmid of claim 10 designated psel-10.8/1A (ATCC Accession No. 209154).

12. A host vector system for the production of a SEL-10

which comprises the vector of claim 9 and a suitable host.

13. The host vector system of claim 12, wherein the suitable host is a bacterial cell, insect cell, plant or animal cell.

14. The host vector system of claim 12, wherein the SEL-10 produced is labeled.

15. A purified wild-type SEL-10 protein or purified wild-type SEL-10 fragment thereof.

16. The purified wildtype SEL-10 protein or fragment thereof which is labeled.

17. A purified mutated SEL-10 protein or purified mutated SEL-10 fragment thereof.

18. The purified mutated SEL-10 protein or fragment thereof which is labeled.

19. A method for production of an antibody capable of binding to wild-type SEL-10 or mutated SEL-10 protein comprising:

a) administering an amount of the purified protein or fragment of wild-type SEL-10 or mutated SEL-10 to a suitable animal effective to produce an antibody against wild-type SEL-10 or mutated SEL-10 protein in the animal; and

b) testing the produced antibody for capability to bind wild-type SEL-10 or mutated SEL-10.

20. The method of claim 19, wherein the antibody is

produced by in vitro immunization.

21. The method of claim 19, wherein the produced antibody is tested by Western blot analysis.

22. The method of claim 19, wherein the produced antibody is tested by immunoprecipitation.

23. The method of claim 19, wherein the produced antibody is tested by staining of cells or tissue sections.

24. A method for production of an antibody capable of binding to wild-type SEL-10 or mutated SEL-10 protein comprising:

a) determining conserved regions revealed by alignment of the wild-type SEL-10 or mutated SEL-10 protein sequences;

b) synthesizing peptides corresponding to the revealed conserved regions;

c) administering an amount of the synthesized peptides to a suitable animal effective to produce an antibody against the peptides in the animal; and

d) testing the produced antibody for capability to bind wild-type SEL-10 or mutant SEL-10.

25. The method of claim 24, wherein the antibodies are produced by in vitro immunization.

26. The method of claim 24, wherein the produced antibody is tested by Western blot analysis.

27. The method of claim 24, wherein the produced antibody

is tested by immunoprecipitation.

28. The method of claim 24, wherein the produced antibody is tested by staining of cells or tissue sections.

29. An antibody capable of specifically binding to wild-type SEL-10 or mutated SEL-10.

30. An antibody produced by the method of claim 19 or 24.

31. The monoclonal antibody of claim 30.

32. A transgenic animal comprising the isolated nucleic molecule of claim 1, 2, 3, 4 or 5.

33. The transgenic animal of claim 32, wherein the animal is a *Caenorhabditis elegans*.

34. A transgenic animal comprising the isolated nucleic acid molecule of claim 2.

35. The transgenic animal of claim 34, wherein the animal is a *Caenorhabditis elegans*.

36. A method for identifying a compound which is capable of ameliorating Alzheimer's disease comprising administering an effective amount of the compound to the transgenic animal of claim 32, 33, 34 or 35, alteration of the conditions of the transgenic animal indicating that the compound is capable of ameliorating Alzheimer's disease.

37. A method for determining whether a compound is capable of ameliorating Alzheimer's disease comprising:

a) treating *Caenorhabditis elegans* mutants having reduced, increased or altered sel-10 activity with the

compound; and

b) determining whether the compound suppresses, enhances or has no effect on the phenotype of the mutants, the suppression or enhancement of the phenotype indicating the compound is capable of ameliorating Alzheimer's disease.

38. A method for determining whether a compound is capable of ameliorating Alzheimer's disease comprising:

a) contacting the compound with a cell which expresses both SEL-12 and SEL-10; and

b) determining whether the compound increases, decreases or has no effect on the amount of SEL-12 in the cell, the increase or decrease of SEL-12 indicating that the compound is capable of ameliorating Alzheimer's disease.

39. A method for determining whether a compound is capable of ameliorating Alzheimer's disease comprising:

a) contacting the compound with a cell which expresses both LIN-12 and SEL-10; and

b) determining whether the compound increases, decreases or has no effect on the amount of LIN-12 in the cell, the increase or decrease of LIN-12 indicating that the compound is capable of ameliorating Alzheimer's

disease.

40. A method for determining whether a compound is capable of ameliorating Alzheimer's disease comprising:

a) contacting the compound with a cell which expresses both mammalian presenilin and SEL-10; and

b) determining whether the compound increases, or decreases or has no effect on the amount of mammalian presenilin in the cell, the increase or decrease of mammalian presenilin indicating that the compound is capable of ameliorating Alzheimer's disease.

41. A method for identifying a compound which is capable of treating cancer comprising administering an effective amount of the compound to the transgenic animal of claim 32, 33, 34 or 35, alteration of the conditions of the transgenic animal indicating that the compound is capable of treating cancer.

42. A method for determining whether a compound is capable of treating cancer comprising:

a) treating *Caenorhabditis elegans* mutants having reduced, increased or altered sel-10 activity with the compound; and

b) determining whether the compound suppresses, enhances or has no effect on the phenotype of the mutants, the suppression or enhancement of the phenotype indicating the compound is

capable of treating cancer.

43. A method for determining whether a compound is capable of treating cancer comprising:

a) contacting the compound with a cell which expresses both LIN-12 and SEL-10; and

b) determining whether the compound increases, decreases or has no effect on the amount of LIN-12 in the cell, the increase or decrease of LIN-12 indicating that the compound is capable of treating cancer.

44. A method for determining whether a compound is capable of treating cancer comprising:

a) contacting the compound with a cell which expresses both SEL-12 and SEL-10; and

b) determining whether the compound increases, decreases or has no effect on the amount of SEL-12 in the cell, the increase or decrease of SEL-12 indicating that the compound is capable of treating cancer.

45. A method for determining whether a compound is capable of treating cancer comprising:

a) contacting the compound with a cell which expresses both mammalian Notch and SEL-10; and

b) determining whether the compound

increases, decreases or has no effect on the amount of mammalian Notch in the cell, the increase or decrease of mammalian Notch indicating that the compound is capable of treating cancer.

46. A method for identifying a suppressor that affects lin-12 or sel-12 activity in the same manner as sel-10, comprising:

a) mutagenizing mutant lin-12 or sel-12 *Caenorhabditis elegans* worms with an effective amount of an appropriate mutagen:

b) screening for revertants in the F1, F2, and F3 generations; and

c) isolating the screened revertants, thereby identifying a suppressor of the phenotype of lin-12 or sel-12 mutation.

47. A suppressor identified by the method of claim 46.

48. A method for identifying an enhancer that affects lin-12 or sel-12 activity in the same manner as sel-10, comprising:

a) mutagenizing mutant lin-12 or sel-12 *Caenorhabditis elegans* worms with an effective amount of an appropriate mutagen:

b) screening for enhancement in the F1, F2, and F3 generations; and

c) isolating the screened enhancers, thereby identifying an enhancer of the phenotype of lin-12 or sel-12 mutation.

49. An enhancer identified by the method of claim 48.

50. A method for producing suppressors of a sel-10 allele comprising:

a) mutagenizing sel-10 mutant hermaphrodites with an effective amount of a mutagen;

b) screening for revertants in the F1, F2, and F3 generations; and

c) isolating the screened revertants.

51. A suppressor produced by the method of claim 50.

52. A method for identifying a suppressor gene comprising performing DNA sequence analysis of the suppressor of claim 51 to identify the suppressor gene.

53. The suppressor gene identified by the method of claim 52.

54. A method for producing enhancers of a sel-10 allele comprising:

a) mutagenizing sel-10 mutant hermaphrodites with an effective amount of a mutagen;

b) screening for enhanced mutants in the F1, F2, and F3 generations; and

c) isolating the enhanced mutants.

55. An enhancer produced by the method of claim 54.

56. A method for identifying an enhancer gene comprising performing DNA sequence analysis of the enhancer of claim 55 to identify the enhancer gene.

57. The enhancer gene identified by the method of claim 56.

58. A method for reversing the malignant phenotype of cells comprising:

a) linking the wild-type or mutated sel-10 gene to a regulatory element such that the expression of the sel-10 gene is under the control of the regulatory element; and

b) introducing the linked sel-10 gene into the malignant cells for the expression of the sel-10 gene so as to reverse the malignant phenotype of cells.

59. The method of claim 58, which further comprises placing the cells from step (b) in appropriate conditions to express the sel-10 gene such that the expression of the sel-10 gene will reverse the transforming phenotype of the malignant cells.

60. The method of claim 59, which further comprises inducing the expression of the sel-10 gene which will reverse the transforming properties of the cells, thereby reversing the phenotype of the

malignant cells in the subject.

61. A pharmaceutical composition comprising an effective amount of the compound identified by the method of claim 36 and a suitable carrier.

62. A pharmaceutical composition comprising an effective amount of the compound identified by the method of claim 41 and a suitable carrier.

63. A method of ameliorating Alzheimer's disease which comprises administrating the pharmaceutical composition of claim 61 in an amount effective to ameliorate Alzheimer's disease.

64. A method of treating cancer which comprises administering the pharmaceutical composition of claim 62 in an amount effective to treat cancer.

FIG. 1A *sel-10* cloning

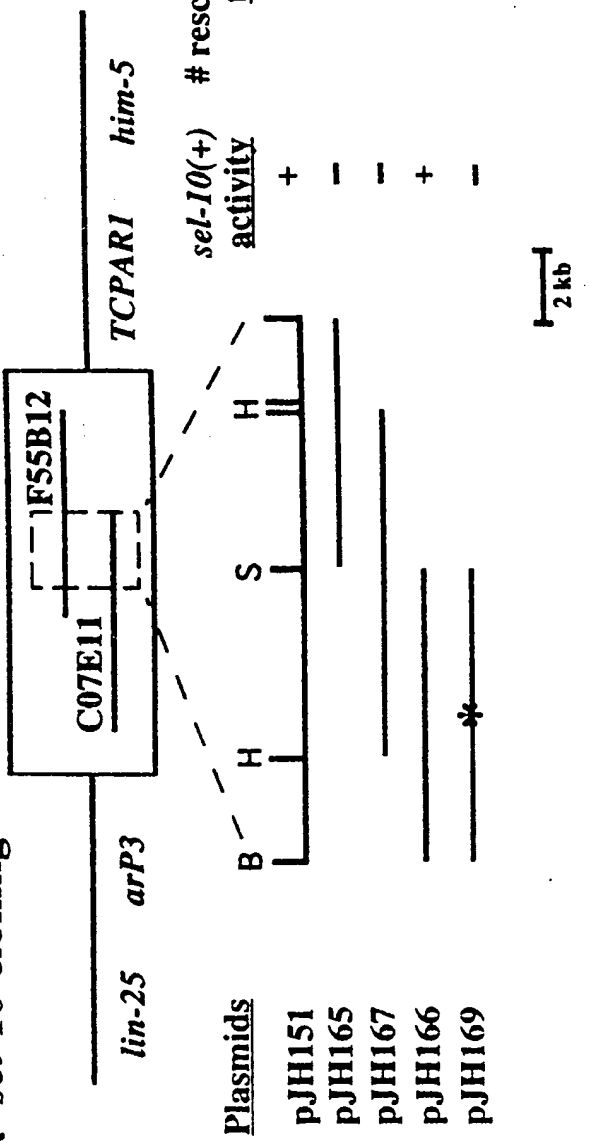

FIG. 1B Predicted *sel-10* transcription unit

FIG. 2A

```
tcatagttttatcgactttccttttgtgttcaaattcttcattcccagtagtttttgtcctttattcatttccaattctttttcagccat

 M  W  P  R  N  D  V  H  M  D  D  G  S  M  T  P  E  D  Q  E  P  V  T  D  N  D  M  E  Y  N    30
ATGTGGCCACGAAATGATGTACACATGGATGATGGATCGATGACACCGGAGGACCAGGAGCCTGTTACCGATAATGATATGGAATATAAT    90

 D  N  G  E  E  S  S  Y  S  N  G  S  S  S  S  Y  N  A  D  K  L  S  S  S  R  P  L  Q  H  K    60
GACAATGGAGAAGAAAGCTCGTACAGCAATGGCTCTTCTTCCAGCTACAATGCTGACAAATTATCGTCTTCCAGACCTTTGCAACACAAA   180

 L  D  L  S  A  S  P  S  R  N  N  D  L  N  P  R  V  E  H  L  I  A  L  F  K  D  L  S  S  A    90
CTTGATTTATCGGCTTCTCCCTCTCGAAACAACGACCTCAATCCGCGTGTCGAACATTTGATCGCATTATTCAAGGATCTATCAAGCGCG   270

 E  Q  M  D  A  F  T  R  L  L  Q  E  S  N  M  T  N  I  R  Q  L  R  A  I  I  E  P  H  F  Q   120
GAACAAATGGATGCATTCACACGTCTGCTTCAGGAATCCAACATGACAAATATTCGACAGCTGCGTGCCATTATTGAACCCCATTTCCAG   360

 R  D  F  L  S  C  L  P  V  E  L  G  M  K  I  L  H  N  L  T  G  Y  D  L  L  K  V  A  Q  V   150
CGTGATTTCCTCTCCTGCCTCCCTGTCGAGCTCGGAATGAAAATCCTTCACAATTTAACCGGATATGACCTGCTCAAAGTGGCACAGGTG   450

 S  K  N  W  K  L  I  S  E  I  D  K  I  W  K  S  L  G  V  E  E  F  K  H  H  P  D  P  T  D   180
TCGAAAAATTGGAAATTGATATCTGAAATTGACAAAATTTGGAAGAGTCTTGGTGTCGAAGAGTTTAAACATCATCCAGATCCCACAGAC   540

 R  V  T  G  A  W  Q  G  T  A  I  A  A  G  V  T  I  P  D  H  I  Q  P  C  D  L  N  V  H  R   210
CGAGTTACTGGTGCGTGGCAAGGAACTGCAATTGCTGCTGGAGTCACTATTCCTGATCACATTCAGCCATGTGATCTTAATGTTCATCGA   630

 F  L  K  L  Q  K  F  G  D  I  F  E  R  A  A  D  K  S  R  Y  L  R  A  D  K  I  E  K  N  W   240
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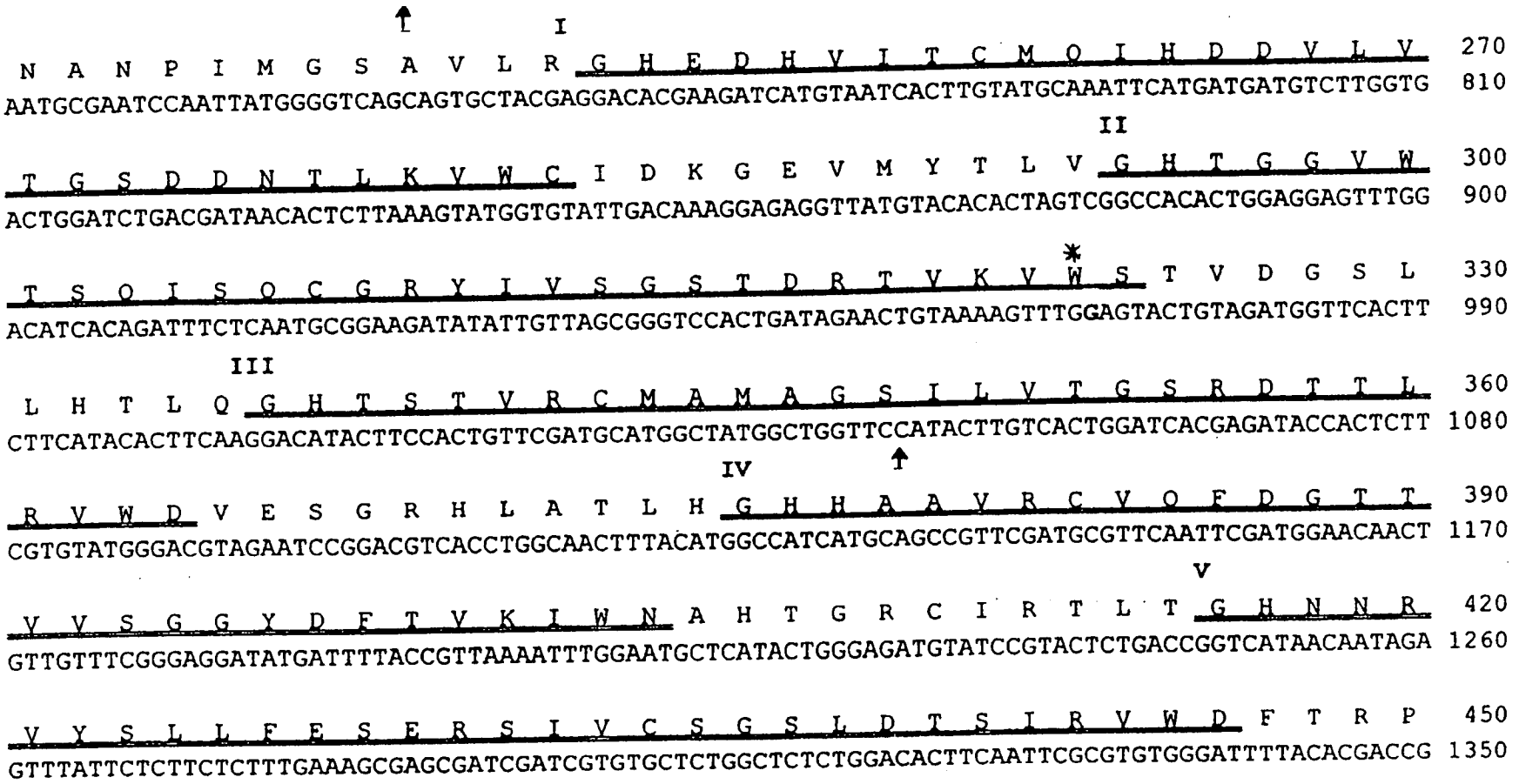
FIG. 2B


I
II
III
IV
V
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T G S D D N T L K V W C I D K G E V M Y T L V G H T G G V W 300
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T S Q I S Q C G R Y I V S G S T D R T V K V W S T V D G S L 330
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L H T L Q G H T S T V R C M A M A G S I L V T G S R D T T L 360
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R V W D V E S G R H L A T L H G H H A A V R C V Q F D G T T 390
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V V S G G Y D F T V K I W N A H T G R C I R T L T G H N N R 420
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V Y S L L F E S E R S I V C S G S L D T S I R V W D F T R P 450
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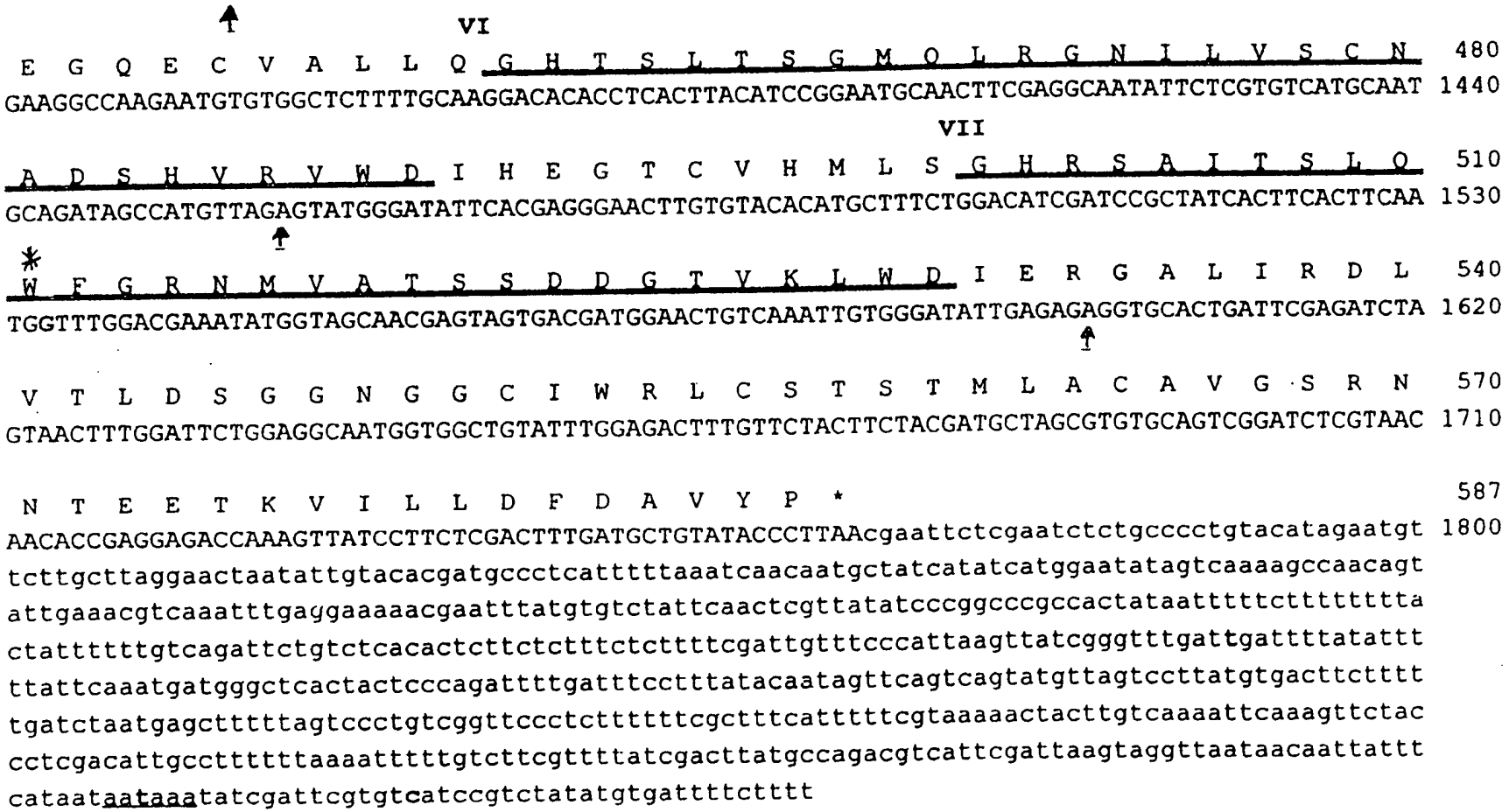
FIG. 2C


VI
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VII
A D S H V R V W D I H E G T C V H M L S G H R S A I T S L Q 510
GCAGATAGCCATGTTAGAGTATGGGATATTCACGAGGGAACTTGTGTACACATGCTTTCTGGACATCGATCCGCTATCACTTCACTTCAA 1530
W F G R N M V A T S S D D G T V K L W D I E R G A L I R D L 540
TGGTTTGGACGAAATATGGTAGCAACGAGTAGTGACGATGGAACTGTCAAATTGTGGGATATTGAGAGAGGTGCACTGATTCGAGATCTA 1620
V T L D S G G N G G C I W R L C S T S T M L A C A V G S R N 570
GTAACTTTGGATTCTGGAGGCAATGGTGGCTGTATTTGGAGACTTTGTTCTACTTCTACGATGCTAGCGTGTGCAGTCGGATCTCGTAAC 1710
N T E E T K V I L L D F D A V Y P * 587
AACACCGAGGAGACCAAAGTTATCCTTCTCGACTTTGATGCTGTATACCCTTAAcgaattctcgaatctctgcccctgtacatagaatgt 1800
tcttgcttaggaactaatattgtacacgatgccctcatttttaaatcaacaatgctatcatatcatggaatatagtcaaaagccaacagt
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cataataataaatatcgattcgtgtcatccgtctatatgtgattttctttt

FIG. 3A

Alignment of F-Box

```
SEL-10   127   LPVELGMKILHNLTGYDLLKVAQVSKNW.KLISEIDKIWKSLG
CDC4     278   LPFEISLKIFNYLQFEDIINSLGVSQNWNKIIRKSISLWKLL
MD6       60   LPLELSFYLKWLDPQTLLTCCLVSKQRNKVISACIEVWQTAC
```

FIG. 3B

Alignment of WD40 repeats

I	SEL-10	253	GHEDHVITCMQIHDDVLVTGSDDNTLKVWC
	CDC4	419	GHMTSVITCLQFEDNYVITGADDKMIRVYD
II	SEL-10	294	GHTGGVWTSQISQCGRYIVSGSTDRTVKVWS
	CDC4	460	GHDGGVWALKYAHGG.ILVSGSTDRTVRVWD
III	SEL-10	336	GHTSTVRCMAMAG....SILVTGSRDTTLRVWD
	CDC4	502	GHNSTVRCLDIVEYKNIKYIVTSGRDNTLHVWK
IV	SEL-10	376	GHHAAVRCVQFDGTTVVSGGYDFTVKIWN
	CDC4	569	GHMASVRTVSGHGNIVVSGSYDNTLIVWD
V	SEL-10	416	GHNNRVYSLLFESERSIVCSGSLDTSIRVWD
	CDC4	609	GHTDRIYSTIYDHERKRCISASMDTTIRIWD
VI	SEL-10	461	GHTSLTSGMQLRGNILVSCNADSHVRVWD
	CDC4	671	GHTALVGLLRLSDKFLVSAAADGSIRGWD
VII	SEL-10	501	GHR..SAITSLQWFGRNMVATSSDDGTVKLWD
	CDC4	710	HHTNLSAITT.FYVSDNILVSGSENQFN.IYN

FIG. 4A

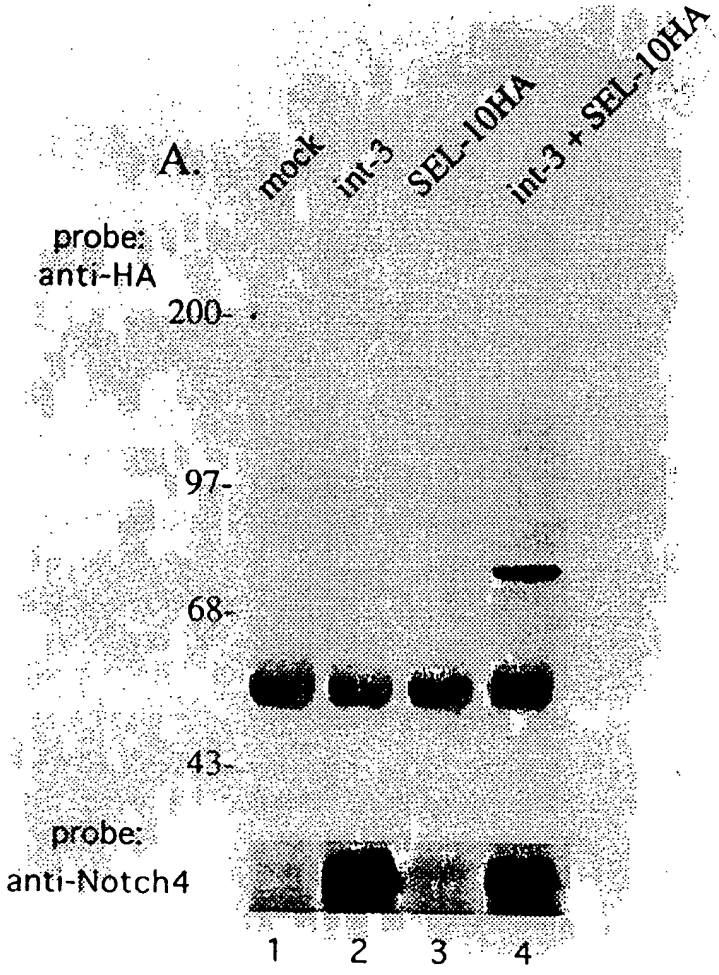

A.
mock
int-3
SEL-10HA
int-3 + SEL-10HA
probe:
anti-HA
200-
97-
68-
43-
probe:
anti-Notch4
1
2
3
4

FIG. 4B

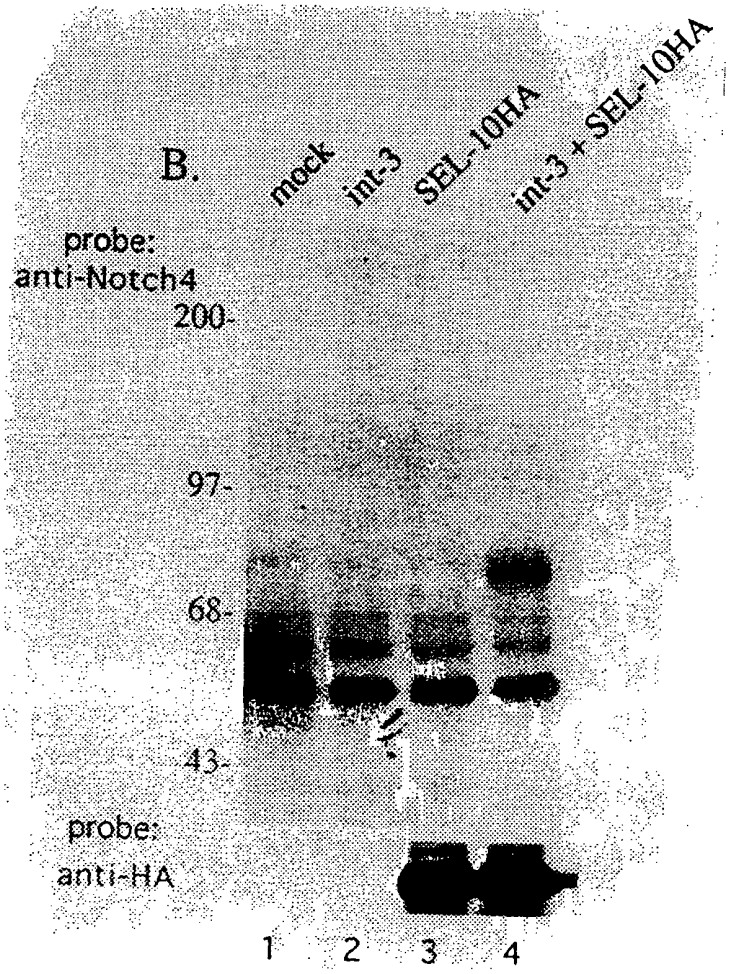
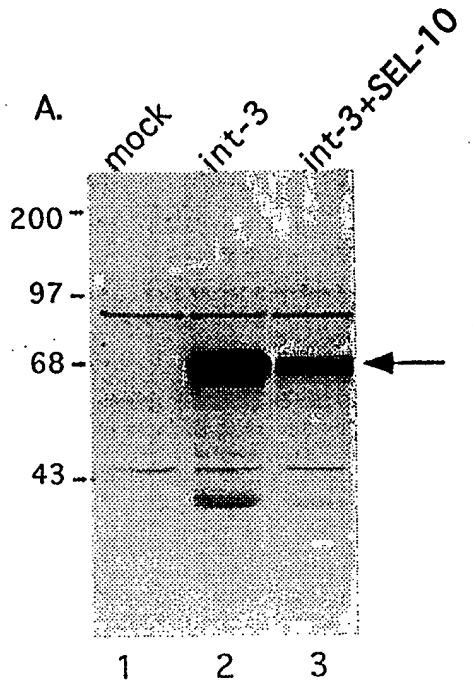

B.
mock
int-3
SEL-10HA
int-3 + SEL-10HA
probe:
anti-Notch4
200-
97-
68-
43-
probe:
anti-HA
1
2
3
4

FIG. 5



INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/15335

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C12P 21/06; C12N 1/20, 15/00; C07H 17/00
US CL :435/69.1, 320.1, 325, 252.3; 536/23.1, 23.5

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/69.1, 320.1, 325, 252.3; 536/23.1, 23.5

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, Dialog

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- A	WILSON, R. et al. 2.2 Mb of contiguous nucleotide sequence from chromosome III of C. elegans. Nature. 03 March 1994, Vol. 368, pages 32-38, see entire document.	1-7 --- 8-14
X ---- A	YOCHEM, J. et al. Structural comparison of the yeast cell division cycle gene CDC4 and a related pseudogene. J. Mol. Biol. 1987, Vol. 195, pages 233-245, see entire document.	1, 6, 7 -------- 2-5, 8-14

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 14 OCTOBER 1998	Date of mailing of the international search report 23 OCT 1998
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer KAREN COCHRANE CARLSON, PH.D. Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/15335

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-14

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/15335

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1.
Group I, claim(s) 1-14, drawn to nucleic acid encoding Sel-10.

Group II, claim(s) 15-18, drawn to Sel-10.

Group III, claim(s) 19-31, drawn to method of making antibodies with Sel-10.

Group IV, claim(s)32-35, drawn to transgenic animals.

Group V, claim(s) 37-40, drawn to method for identifying compounds for ameliorating Alzheimer's Disease.

Group VI, claim(s) 42-45, drawn to a method for identifying compounds capable of treating cancer.

Group VII, claim(s) 46, drawn to a method for identifying suppressors of lin12 or Sel-12.

Group VIII, claim(s) 47, drawn to suppressor of lin12 or sel-12.

Group IX, claim(s) 48, drawn to a method for identifying enhancers of lin12 or sel-12.

Group X, claim(s) 49, drawn to enhancers of lin12 or sel-12.

Group XI, claim(s) 50, drawn to a method for producing a suppressor of Sel-10.

Group XII, claim(s) 50, drawn to a suppressor of Sel-10.

Group XIII, claim(s) 52, drawn to a method for identifying a gene encoding a suppressor of Sel-10.

Group XIV, claim(s) 53, drawn to a gene encoding a suppressor of Sel-10.

Group XV, claim(s) 54, drawn to a method for producing an enhancer of Sel-10.

Group XVI, claim(s) 55, drawn to an enhancer of Sel-10.

Group XVII, claim(s) 56, drawn to a method for identifying a gene encoding an enhancer of Sel-10.

Group XVIII, claim(s) 57, drawn to a gene encoding an enhancer of Sel-10.

Group XIX, claim(s) 58-60, drawn to a method for reversing malignancy via antisense therapy.

The inventions listed as Groups I-XIX do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

The products of Groups I, II, IV, VIII, X, XII, XIV, XVI, and XVIII differ in structure and function and therefore these products lack the same or corresponding special technical features.

The nucleic acid of Group I is not used in the methods of Groups III, V-VII, IX, XI, XIII, XV, XVII, or XIX. Therefore, Group I does not provide a special technical feature between Groups III, V-VII, IX, XI, XIII, XV, XVII, and XIX.